The phenotypic and functional characterisation of regulatory T-cells from patiends with end stage liver disease; Implications for adoptive cell therapy

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The Phenotypic and Functional Characterisation of Regulatory T cells from Patients with End Stage Liver Disease; Implications for Adoptive Cell Therapy

A thesis submitted to King's College London for the degree of Doctor of Philosophy

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DECLARATION

The work described in this thesis was carried out at the Immunoregulation Laboratory, King's College London. Except where acknowledged, the data presented is my own original work.

ABSTRACT

The adoptive transfer of human regulatory T cells (Tregs) in transplantation offers an attractive therapeutic alternative in the current struggle to improve long-term outcomes.

$CD4^+CD25^+FOXP3^+$ (Tregs) play an important role in immunoregulation and have been shown in animal models to promote transplantation tolerance. Phase I trials in bone marrow transplantation and type I diabetes have already shown that *ex vivo* expanded Tregs have an excellent safety profile, which is encouraging for the broader application of these cells. The clinical trials initiated at King's College London, ThRIL and the ONE study, are the leading trials of autologous Treg immunotherapy worldwide in the setting of liver and kidney transplantation, respectively. The success of these trials is reliant on the implementation of protocols that comply with Good Manufacturing Practice (GMP) guidelines centered on the successful isolation and expansion of a functional and stable human Treg population from prospective transplant recipients.

The main focus of this thesis has been the adoptive cell therapy of Tregs in the context of liver transplantation. In this regard, it was first pertinent to study the biology of Tregs from patients with alcohol related cirrhosis (ARC), representing the majority of patients on the liver transplant waiting list. As such, an in-depth phenotypic and functional characterisation of the isolated Tregs from these patients was carried out. The results shown herein demonstrate that Tregs from ARC patients display impaired suppressive function. Based on this finding, a series of experiments were conducted in order to delineate the mechanism behind the apparent defect in Treg suppressive function. This led to a novel discovery of a defect in the expression

of the cytoprotective enzyme, heme oxgenase-1 (HO-1), by patient Tregs, in correlation with the apparent Treg dysfunction.

Subsequently, adherence to a GMP compatible 36-day expansion protocol resulted in the enrichment of a pure population of Tregs (91.3% $CD4^+CD25^+$ and 0.153% $CD8^+$ cells), reaching numbers needed for their clinical translation. In addition, the protocol ensured the maintenance of FOXP3 expression (94.6% of the $CD4^+CD25^+$ cells expressed FOXP3 at the end of expansion) with an increase in the frequency of $FOXP3^{Hi}$ cells throughout expansion. Culture in the presence of rapamycin also confirmed the stability of the expanded Tregs, whereby the cells did not convert to Th17 cells when cultured in the presence of pro-inflammatory stimuli.

More recently, in the context of solid organ transplantation, the superiority of murine Treg lines with specificity for the graft as compared to polyspecific Tregs, in the protection against allograft damage, has come to light. Here, further evidence was provided, using a humanized mouse model of skin transplantation, demonstrating that human Tregs with direct allospecificity are more potent, compared to polyclonal Tregs, at averting alloimmune mediated skin damage. The data further indicated that allogeneic B cells were highly effective antigen presenting cells in this setting and concluded that a fundamental prerequisite precluding this as a successful therapeutic option was the requirement of a highly pure population of Tregs prior to allospecific *ex vivo* expansion.

The practicality of Treg adoptive cell therapy is now widely accepted, provided that tailor-made clinical grade procedures for the isolation and *ex vivo* cell handling are available. The work presented in this thesis has addressed the prospects of Treg cell therapy, whilst proposing further mechanistic data concerning Treg biology.

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To my loving family

'For you are my strength and my weakness'.

TABLE OF CONTENTS

TABLE OF FIGURES

Chapter 1 Introduction

Chapter 2 : Materials and Methods

Chapter 3 : An In-depth Phenotypic and Functional Characterisation of Regulatory T Cells from Patients with Alcohol-related Cirrhosis

Chapter 4 : *Ex vivo* Expansion of Regulatory T cells from Patients with End Stage Liver Disease; Increased Suppressor Function and Clinical Application

Chapter 6 : General Discussion

Appendices

LIST OF TABLES

Chapter 2 : Materials and Methods

Appendices

LIST OF ABBREVIATIONS

A

ADP	Adenosine Diphosphate
ALD	Alcoholic Liver Disease
ALP	Alkaline Phosphatase
APC	Allophycocyanin
APC	Antigen Presenting Cell
ALT	Alanine Aminotransferase
AMP	Adenosine Monophosphate
AOPP	Advanced Oxidation Protein Product
ARC	Alcohol-Related Cirrhosis
ARE	Antioxidant Response Element
AST	Aspartate Aminotransferase
ATG	Anti-Thymocyte Globulin
ATP	Adenosine Triphosphate

B

Bach 1	BTB and CNC Homologue
Bcl6	B cell Lymphoma-6
BCR	B cell Receptor
BMT	Bone Marrow Transplantation
BR	Bilirubin
Breg	Regulatory B cells
BSA	Bovine Serum Albumin

C

cAMP	Cyclic Adenosine Monophsophate
CCR	Chemokine Receptor
CD	Cluster of Differentiation

CDR	Complementarity-Determining Region
CD62L	62 ligand/L-Selectin
CFSE	Carboxyfluorescein Succinimidyl Ester
CLP	Common Lymphoid Progenitors
CNI	Calcineurin Inhibitor
CO	Carbon Monoxide
CRF	Clinical Research Facility
CTL	Cytotoxic T cell
CTLA-4	Cytotoxic T-Lymphocyte Antigen-4
CXCR3	CXC Chemokine Receptor 3

D

DC	Dendritic Cell
DMSO	Dimethyl Sulphoxide
DN	Double Negative
DP	Double Positive

E

EDTA	Ethylenediaminetetraacetic Acid
EF450	eFluor450
ELISA	Enzyme Linked Immunosorbent Assay
ERK	Extracellular-Regulated Kinase

F

FACS	Fluorescence Activated Cell Sorter
FasL	Fas Ligand
FCS	Fetal Calf Serum
Fe	Ferrous Iron
FITC	Fluorescin Isothiocyanate
FOXP3	Forkhead Box P3
FSC	Forward Scatter

G

GARP	Glycoprotein A Repetitions Predominant
GGT (γGT)	Gamma-Glutamyl Transpeptidase
GITR	Glucocorticoid-Induced TNFR Family Related Gene
GMP	Good Manufacturing Practice
GSK3β	Glycogen Synthease Kinase-3β
GvHD	Graft versus Host Disease

H

4-HNE	4-Hydroxynonenal
H_2SO_4	Sulphuric Acid
Hb	Haemoglobin
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
HC	Healthy Control
HDAC	Histone Deacetylase
Hi	High
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HO-1	Heme Oxgenase-1
HS	Human Serum
HSC	Haematopoietic Stem Cell

I

ICER	Inducible cAMP Early Repressor
ICOS-L	Inducible T-cell Costimulator Ligand
IDO	Indoleamine 2, 3-Dioxygenase
iTreg	Induced Regulatory T cell
IFN	Interferon

IFN-γ	Interferon Gamma
IgG	Immunoglobulin G
IL	Interleukin
INR	International Normalized Ratio
IPEX	Immunodysregulation, Polyendocrinopathy, Enteropathy, X-linked Syndrome
IRF4	Interferon Regulatory Factor-4
IS	Immunosuppression
Int	Intermediate
iTreg	'Induced Treg'

K

Keap1	Kelch-like ECH-Associated Protein 1

L

L	Ligand
Lo	Low
LPS	Lipopolysaccharide
LT	Liver Transplantation

M

MDSC	Myeloid-Derived Suppressor Cells
MELD	Model for End Stage Liver Disease
MFI	Mean Fluorescent Intensity
mHAg	Minor Histocompatibility Complex Antigens
MHC	Major Histocompatibility Complex
MLR	Mixed Leukocyte Reaction
MMF	Mycophenolate Mofetil
Mreg	Regulatory Macrophage
MSC	Mesenchymal Stromal Cells
mTOR	Mammalian Target Of Rapamycin

mTORC1	Mechanistic target of rapamycin complex 1

N

NaCl	Sodium Chloride
NAFLD	Non-Alcoholic Fatty Liver Disease
NASH	Non-Alcoholic Steatohepatitis
NFAT	Nuclear Factor Activated T cells
NK	Natural Killer Cells
NOD	Non-Obese Diabetic
Nrf2	NF-E2-Related Factor 2
Nrp-1	Neuropilin

P

PB	Pacific Blue
PBC	Primary Biliary Cirrhosis
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
pDC	Plasmocytoid DCs
PD-1	Programmed Cell Death-1
PDL-1	Programmed Cell Death Ligand 1
PE	Phycoerythrin
PerCP	Peridinin-Chlorophyll-Protein Complex
PI3-K	Phophatidylinositol-3 Kinase
PMA	Phorbol 12-Myristate 13-Acetate
pTreg	Peripheral Treg

R

Rag	Recombination-Activating-Gene
RAPA	Rapamycin
RIPA	Radio-Immunoprecipitation Assay

RORC	Retinoic Acid-Related Orphan Receptor
ROS	Reactive Oxygen Species

S

S6K1	Protein S6 Kinase-1
S	Stimulation
SCID	Severe Combined Immunodeficiency
SD	Standard Deviation
SEM	Standard Error of the Mean
SP	Single Positive
SSC	Side Scatter
STAT	Signal Transducers and Activators of Transcription

T

T1D	Type-1 Diabetes
T-bet	T-cell-Specific T-box Transcription Factor
TCR	T Cell Receptor
Teff	$CD4^{+}CD25^{-}$ T Effector
TF	Transcription Factor
Tfh	T Follicular Helper
TGF-β	Transforming Growth Factor Beta
Th	T helper Cell
Thp	Pluripotent Naïve T Cell
Th1	T helper Cell 1
Th2	T helper Cell 2
Th17	T helper Cell 17
ThRIL	Tregs in Liver
TIM-3	T cell Immunoglobulin and Mucin Domain-3
TLR	Toll-Like Receptor
TMB	3,3',5,5'– Tetramethylbenzidine

TNFα	Tumor Necrosis Factor-α
TRAIL	Tumour Necrosis Factor-Related Apoptosis-Inducing Ligand
Treg	Regulatory T cell
tTreg	Thymic Derived Regulatory T cell
TSDRs	Treg-Specific Demethylation Region

U

UCB	Umbilical Cord Blood

V

V	Variable

Z

ZnPP	Zinc Protoporphyrin

Chapter 1

INTRODUCTION

1.1 TRANSPLANTATION

The introduction of T-cell directed immunosuppressive agents in the clinical transplantation of solid organs has advanced remarkably and is currently a well-established treatment for end stage failure of several major organs. However, despite vast improvements in short-term survival rates, long-term survival remains poor owing to episodes of chronic rejection and the relative toxicity associated with life-long immunosuppression (Meier-Kriesche et al. 2004). Thus, the definitive goal of transplantation is to achieve 'tolerance', a state in which the host's immune system can be reprogrammed and subsequently directed to accept a transplant without the need for long-term immunosuppression.

In this pursuit, a thorough understanding of the immune mechanisms implicated in transplant allograft rejection is essential so as to better dictate an approach to tolerance induction.

1.1.1. ALLORECOGNITION AND THE ALLORESPONSE – DEFINITIONS

In an immunocompetent host, transplantation of an organ from a genetically identical ("syngeneic") individual does not lead to immunological reactivity directed against the graft, in contrast to transplantation between genetically disparate, or "allogeneic", individuals. Allorecognition is the term used to refer to identification of tissues of allogeneic origin by a recipient's immune system through the engagement of a receptor-ligand system. As such, the T and B cell receptors (TCR and BCR respectively) are key to recognising tissue–expressed genetically encoded polymorphisms (ligands) between members of the same species. These

polymorphisms, determining compatibility of tissues (histocompatibility), can be divided into major (class I and II) and minor histocompatibility complex antigens (MHC and mHAg respectively).

Receptor-ligand (MHC-TCR/BCR) interactions between histoincompatible individuals result in the initiation of an immune response, referred to as the "alloresponse". The alloresponse in non-tolerant patients and in the absence of immunosuppression invariably leads to graft rejection. This is characterised by tissue inflammation, architectural distortion and infiltration by graft-reactive T cells with effector function into the graft. The vigor of alloresponsiveness is reflected *in vitro* by the mixed leukocyte reaction (MLR) and *in vivo* by the rapidity, provoked by MHC incompatibility, of early transplant rejection (Sherman and Chattopadhyay 1993).

However, it is noteworthy to mention, that allorecognition does not always result in rejection of donor grafts. Indeed, in clinical organ transplantation, particularly following liver transplantation, normal allograft function has been reported despite complete discontinuation of all immunosuppressive drugs (Takatsuki et al. 2001, Lerut and Sanchez-Fueyo 2006, Tisone et al. 2006). Patients spontaneously accepting their grafts are conventionally considered as "operationally" tolerant and provide a proof of concept that immunological tolerance can actually be attained in humans. Furthermore, in experimental transplant models spontaneous transplant tolerance across a full class I/ class II MHC barrier has been observed in outbred pigs (Calne et al. 1969) as well as in a number of fully allogeneic rat strain combinations (Kamada 1985). These divergent responses to transplanted tissues allow a distinction to be

made between “antigenicity”, referring to the likelihood of foreign peptide or MHC molecule immunological recognition, and “immunogenicity”, the capacity to elicit a destructive immune response.

In the following sections the pathways of allorecognition will be reviewed with relevance to the activation of effector T-cell responses, inevitably provoking graft rejection.

1.1.2. PATHWAYS OF ALLORECOGNITION

Three pathways of allorecognition have been described to date **(Figure 1.1)**: the direct pathway, in which recipient T cells recognise intact allogeneic MHC-peptide complexes expressed by foreign cells, (Warrens et al. 1994, Whitelegg and Barber 2004) the indirect pathway, whereby T cells recognise peptides derived from allogeneic MHC proteins presented by self- antigen-presenting cells (APC) (Lechler and Batchelor 1982) and the semi-direct pathway, where recipient dendritic cells (DC) acquire intact allogeneic MHC-peptide complexes from donor cells and present them to recipient T cells (Herrera et al. 2004).

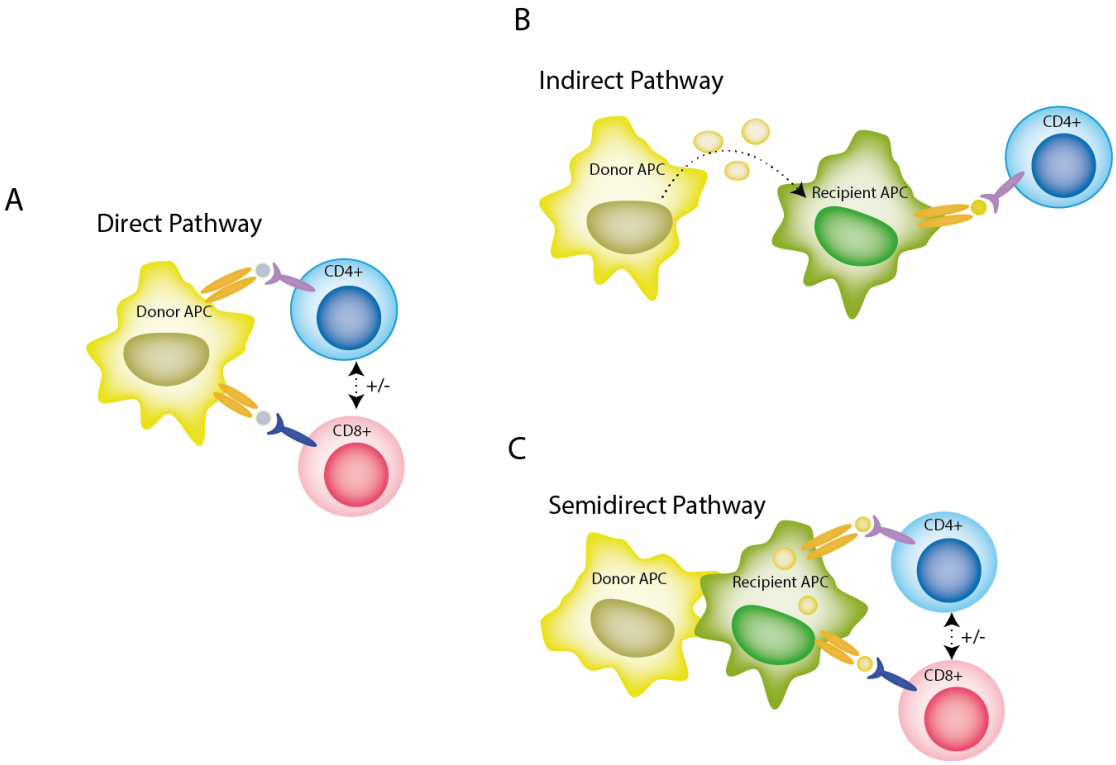


FIGURE 1.1. DIRECT, INDIRECT, AND SEMIDIRECT PATHWAYS OF ALLORECOGNITION.

A. In the direct pathway, intact MHC on donor APCs is recognized directly by recipient $CD4^+$ and $CD8^+$ T cells. **B.** The indirect pathway is characterized by recipient APC uptake of allogeneic donor MHC that has been shed through apoptosis or necrosis. This is then processed, resulting in presentation of donor antigens in the context of recipient MHC class II to recipient $CD4^+$ T cells. **C.** Semidirect allorecognition results from the transfer of cellular membrane components, including intact donor MHC, from donor APCs to recipient APCs. This process may occur through mechanisms such as cell–cell contact or through the transfer of donor exosomes that fuse with recipient APC cell membranes. Recipient APCs are then chimeric for MHC, and are able to stimulate both $CD4^+$ and $CD8^+$ recipient T cells. *Abbreviations: APC- Antigen presenting cell; CD-cluster differentiation; MHC- major histocompatibility complex. Adapted from (Safinia et al. 2010).*

1.1.2.1. DIRECT ALLORECOGNITION

Data available by the end of the 1950s suggested that leukocytes inherently associated with the transplanted tissue were the major source of tissue immunogenicity (Snell 1957). These findings implicated 'passenger' leukocytes as the major immunogenic component of the allograft (Talmage et al. 1977). In support of this, studies looking into the effects of depleting donor bone marrow-derived 'passenger' leukocytes by *in vitro* culture of thyroid (Talmage et al. 1976) or pancreatic islet (Bowen et al. 1980) allografts showed prolonged graft survival. Data to support that DCs are the principal APCs mediating the priming of naïve T-cells against graft antigens was conclusively demonstrated in 1982 by Lechler and Batchelor after it was shown that acceptance of rat renal allografts could be achieved by "parking" the kidney temporarily in an intermediate immunosuppressed recipient, leading to depletion of donor DCs before re-transplantation (Lechler and Batchelor 1982).

T cells with direct allospecificity have a clear role in transplant rejection as demonstrated in a study by Gill's group (Pietra et al. 2000). Here, immunodeficient severe combined immunodeficiency (SCID) or recombination-activating-gene-deficient (*Rag*$^{-/-}$) mice were reconstituted with syngeneic CD4^{+} T cells and were subsequently found to reject MHC class II-expressing cardiac allografts, but not MHC class II-deficient grafts. Furthermore, *Rag*$^{-/-}$MHC class II$^{-/-}$ mice rejected allogeneic cardiac transplants when reconstituted with CD4^{+} T cells. Given that these mice have no CD8^{+} T cells and lack the capacity for self-MHC class II-restricted indirect allorecognition (see below), these results clearly indicate that direct pathway CD4^{+} T cells were both necessary and sufficient to mediate allograft rejection.

The strength of alloresponses triggered by the direct pathway is due in part to the very high frequency of T cells with direct allospecificity (Baker et al. 2001). There is evidence to indicate that this mode of allorecognition results from cross-reactivity by T cells specific for a self MHC molecule, 'A', with peptide 'x' on an allogeneic MHC molecule, 'B', with peptide 'y' (Rogers and Lechler 2001). In support of the cross-reactive hypothesis it was evident that a large fraction of the direct alloresponse was derived from T cells with a memory phenotype, indicating that these cells had been primed against foreign antigens in the context of self-MHC molecules (Lombardi et al. 1990, Merkenschlager et al. 1991). Two models have been proposed to account for the high precursor frequency of alloreactive T cells within the direct pathway of allorecognition. These are the 'high determinant density' (Crispe et al. 1986, Baldwin et al. 2004) and 'multiple binary complex' (Matzinger and Bevan 1977) models.

The high determinant density model proposes that alloreactive T cell receptors are able to directly recognise the exposed amino acid polymorphisms on intact foreign MHC and that the nature of the bound peptide is of secondary relevance (Warrens et al. 1994). Thus, if every MHC molecule on a cell's surface can serve as a ligand for an allospecific T cell, this creates a high ligand density, implying that receptors with lower affinity can respond to the foreign MHC, leading to a higher frequency of alloreactivity. In support of this, several studies have shown inhibition of alloresponses after blocking TCR-contacting regions of allo-MHC using synthetic peptides and site-specific mutations, presumably through inhibition of TCR-MHC contact (Schneck et al. 1989, Lombardi et al. 1991, Villadangos et al. 1994). In addition, previous work has shown alloreactivity in the absence of peptide by analyzing the reactivity of several H-2K^{b-}alloreactive cytotoxic T cell (CTL) clones

against H-2K^b-expressing target cells, with and without acid treatment of target cells to remove bound peptide form the MHC binding groove.

The multiple binary complex model proposes that the specificity of the alloreactive TCR for recognition of peptide (derived from a normal cellular or serum protein) bound by allogeneic MHC is of primary importance (Sherman and Chattopadhyay 1993). This model is the equivalent of TCR recognition of conventional self-MHC restricted immune responses to foreign antigen (crystallographic studies over the last decade have provided significant insight into these unique recognition events). Differences in the allo-MHC peptide-binding groove causes binding of a different set of peptides from those of the self-MHC homologue, therefore each peptide-allo-MHC complex is recognised by a different alloreactive T cell and any one MHC mismatch will be able to stimulate a large number of diverse T cells responsive to different antigens. In support of this, displacement of endogenous peptides from allogeneic antigen presenting cells (APCs) by incubation with exogenous peptides leads to loss of allorecognition by allospecific T cells (Eckels et al. 1988). Furthermore, adding peptides, naturally processed and derived from a serum or cellular protein, has been shown to restore allorecognition, and many alloreactive T-cell clones exhibit selective peptide recognition, indicating that they are peptide specific (Rotzschke et al. 1991, Barber et al. 1995, Kuzushima et al. 1995).

In practice, it is probable that both the high determinant density and multiple binary complex mechanisms contribute to direct allorecognition, the overall contribution of each being related to the site and magnitude of the structural differences in the MHC molecules between responder and stimulator cells.

The high frequency of direct alloreactivity suggests that it is likely to dominate priming of direct pathway alloreactive T cells in the immediate post-transplant period. This is supported by markedly elongated transplant survival in direct pathway-incompetent animals. However, depletion of donor-derived DC through apoptosis and elimination by recipient immune reactivity is a process that begins shortly after engraftment. This is accompanied by a decline in frequency of recipient T cells with direct anti-donor allospecificity with time (Hornick et al. 1998), most pronounced in the $CD4^+CD45RO^+$ (memory) subset, consistent with the proposal that it is an encounter with co-stimulation deficient graft parenchymal cells that leads to the fall in frequency (Baker et al. 2001). These observations suggest that the crucial period for activation of recipient direct pathway memory T cells is the first few weeks following transplantation in response to donor DCs.

1.1.2.2. INDIRECT ALLORECOGNITION

Indirect allorecognition was described as a separate entity over 20 years ago (Lechler and Batchelor 1982) following observations that passenger leukocyte-depleted rat renal allografts, weakly immunogenic via the direct pathway, were rejected albeit with slower kinetics than seen with direct responses. It was suggested that conventional antigen presentation pathways for nominal antigens could be responsible for mediating graft rejection in this setting. The "indirect" pathway model proposed that recipient DCs traffic through the graft, take up soluble MHC alloantigens and dead donor cells and process and present alloantigens as peptide on self MHC class II molecules to $CD4^+$ cells in local lymphoid tissue. In support of this, when $H2^K$ DCs (expressing the H2-E molecule) were injected into $H2^b$ recipients (H2-E negative),

within two days the majority of recipient DCs in the draining lymph nodes could be stained by an antibody recognising a complex of H2-A^b with a peptide from H2-Ea chain, indicating that H2-E molecules have been processed by trafficking host DCs and presented as peptides on self-MHC (Inaba et al. 1998). Indeed, MHC class I-deficient recipients, that lack $CD8^+$ T cells are still capable of rejecting MHC class II negative grafts, through indirect presentation of peptides of graft-derived MHC class I to recipient $CD4^+$ T cells (Auchincloss et al. 1993). *In vitro* studies have confirmed the presentation of donor MHC peptides by recipient APCs for both class I (Essaket et al. 1990) and class II (de Koster et al. 1989) peptides and T cell recognition of MHC class I and II antigens in the context of self-MHC class I has also been demonstrated (Kievits and Ivanyi 1991). *In vivo,* processing and presentation of MHC-derived peptides appear to be common physiological events (Hunt et al. 1992) and self restricted T helper cells with indirect allospecificity can provide help for CTL induction during allograft rejection (Lee et al. 1994). In this model, recipient mice, depleted of $CD8^+$ T cells (by *in vivo* treatment with anti-CD8 monoclonal antibody) and subsequently grafted with MHC class II deficient allogeneic skin, rejected the skin grafts rapidly through *de novo* generation of graft-reactive $CD8^+$ CTL. These $CD8^+$ CTL required *in vivo* help from indirect pathway-sensitized $CD4^+$ cells. These results provide evidence that indirect recognition can provide effective help for CTL induction during graft rejection, even when the cytotoxic T cells are sensitized by determinants expressed only on the donor graft.

The decline in direct pathway responses with time is as pronounced in patients with chronic rejection as those with stable graft function, indicating that the direct pathway of allorecognition is not as important for chronic rejection. The indirect alloresponse, on the other hand, founded on antigen capture and processing, is less rapid as

compared to the direct pathway and dominates reactivity to transplanted antigens in the long-term. The requirement for antigen processing and presentation in the context of self-MHC class II means that the indirect pathway is largely dominated by $CD4^+$ T cells. The importance of the indirect pathway to transplant rejection is demonstrated by experimental systems whereby immunization with peptides of allogeneic MHC (eliciting only indirect pathway responses) is sufficient to mediate transplant rejection (Fangmann et al. 1993). Similarly, donor-specific hyporesponsiveness can be created by intrathymic injections of MHC peptides through down-modulation of indirectly-responsive T cells (Sayegh et al. 1994).

Indirectly alloresponsive $CD8^+$ T cells are activated through "cross-priming", whereby APCs process donor proteins, including alloantigens, and present them in peptide form in the context of self MHC class I molecules (Valujskikh et al. 2002). Interestingly, a recent study showed that recipient endothelium was able to present antigens of skin grafts in the context of recipient MHC class I molecules to TCR-transgenic $CD8^+$ T cells, and this indirect $CD8^+$ pathway could result in an IFN-γ-dependent skin graft rejection. However, the significance of this pathway of indirect recognition remains unclear since it has been found that the indirect pathway involving $CD8^+$ cells has no effect on rejection or tolerance of vascularised cardiac allografts (Valujskikh et al. 2006).

Despite dominant focus on host DCs and macrophages when elucidating mechanisms of indirect alloantigen presentation (Steinman 1991, Sayegh and Carpenter 1996), it has been noted that B cells have their own a special niche in indirect alloresponses,

due to the importance of T:B cell interactions. In this regard, B cells are known to constitutively express MHC class II and costimulatory molecules, which enable them to act as potent APCs for alloreactive CD4 T cell activation (Janeway et al. 1987, Ron and Sprent 1987, Wilson et al. 1995). Moreover activated alloreactive B cells, by virtue of their antigen- specific BCR, have the capacity to selectively concentrate specific antigens, making them a repository of alloantigen- presenting APCs. Indeed, the presence of circulating, class-switched, donor-specific antibody suggests that indirect antigen presentation to T cells has occurred via B cells that have captured alloantigen through their surface BCR. T cell help, in turn, then ensures that B cells produce high affinity, class-switched alloantibodies (Steele et al. 1996, Taylor et al. 2007). In murine models of cardiac transplantation, containing targeted deficiencies of MHC class II-mediated Ag presentation confined to the B cell compartment, absent B cell-mediated Ag presentation disrupts both alloantibody production and the progression of $CD4^+$ T cell activation, leading to prolonged cardiac allograft survival (Noorchashm et al. 2006). These findings demonstrated that indirect alloantigen presentation by the recipients' B cells plays an important role in the efficient progression of acute vascularised allograft rejection. These experiments support the growing appreciation of B cells and T:B cell interactions in chronic rejection.

1.1.2.3. THE SEMI-DIRECT PATHWAY

The traditional model of cross-talk between $CD4^+$ and $CD8^+$ T cells during the generation of an immune response relies on the “three-cell” or “linked” model (Ridge et al. 1998). In this model, the generation of pathogen-specific cytotoxic $CD8^+$ T cells requires help from $CD4^+$ T cells activated by the same APC. Observations in

transplantation, however, present an apparent paradox in this model as cross-talk between the direct and indirect pathways (e.g. indirect pathway $CD4^+$ T cells amplifying or regulating direct pathway-responsive $CD8^+$ T cells) necessitate a "four cell" or "unlinked" model (i.e. that each T cell type is activated by a different APC).

The 'semi-direct' pathway (Herrera et al. 2004) **(Figure 1.1)** resolves the four cell problem by stipulating that intact surface donor MHC:peptide complexes are acquired by recipient APCs either through a cell-cell contact (Game et al. 2005) or via exosomes, small vesicles produced by cells in a non-haphazard fashion (Morelli et al. 2004). In this way, recipient DCs acquire and present intact donor MHC class I molecules to direct pathway $CD8^+$ T cells, as well as internalised and processed donor MHC molecules as peptides to $CD4^+$ T cells with indirect allospecificity (Brown et al. 2011). Therefore, presentation to both direct and indirect pathway-responsive T cells occurs in a linked or three-cell manner.

Although there is no direct evidence for an *in vivo* role of this pathway in the context of allograft rejection, it does provide an alternative explanation for several findings that are difficult to understand if only the direct and indirect justifications of allorecognition apply. For instance, embryonic thymic epithelium, naturally devoid of APCs, can be acutely rejected in the absence of the indirect pathway, a phenomenon difficult to explain without the semi-direct pathway of allorecognition (Pimenta-Araujo et al. 2001). Similarly, recipients lacking the indirect pathway (MHC class II-deficient) reject co-stimulation deficient ($CD80^{-/-}CD86^{-/-}$) allogeneic cardiac grafts as

rapidly as wild-type transplants (Mandelbrot et al. 2001). This can be explained by the semi-direct presentation of MHC from the graft rather than trans co-stimulation.

1.1.3. THE ALLORESPONSE AND EFFECTOR CELLS

The central determinant of alloresponsiveness, whether recognition occurs through the direct, indirect or semi-direct pathways, is the APC, which readies recipient T cell responses. In this setting, immunological priming is most likely to occur in secondary lymphoid tissues. In support of this, donor DCs from cardiac allografts migrate to recipient spleen after transplantation (Larsen et al. 1990) and mice lacking secondary lymphoid organs are unable to mount immune responses against transplanted tissues (Lakkis et al. 2000). Additionally, using TCR-transgenic mice it can be appreciated that adoptively transferred $CD4^+$ T cells with direct allospecificity expand initially in the draining lymph nodes and spleen in response to the presence of the alloantigen on transplanted skin. The expanded $CD4^+$ T cells upregulate CD69 and CD25 expression and produce effector cytokines, but downregulate CD45RB and CD62L expression, hallmarks of an effector and/or memory phenotype. After clonal expansion, they infiltrate the graft and this correlates with the timing of graft rejection (Reed et al. 2003, Sandner et al. 2003).

Following alloantigen recognition, in the absence of mitigating immune mechanisms or pharmacological intervention with immunosuppressive drugs, intense infiltration of lymphokine-secreting alloreactive T cells occurs in the graft, inducing the expression of MHC class II on endothelial and epithelial cells, conferring the ability to present antigen to $CD4^+$ T cells (Bal et al. 1990). As passenger leucocytes are depleted with time post-transplantation, endothelial and epithelial cells remain as the only graft-

derived cells expressing donor MHC class II. Direct pathway presentation through these non-professional APCs results in the deletion of naïve T cells, silencing and/or switching to a Th2 phenotype (in antigen-specific T-cell clones), and anergy in memory cells with failure to secrete interleukin (IL) 2 (Lombardi et al. 1997, Marelli-Berg et al. 1997). The lack of proliferation of alloreactive T cells can be overcome by the addition of B7 (CD80/CD86) – expressing cells, suggesting that non-professional APCs lack effective co-stimulatory ability (Marelli-Berg et al. 1996).

The requirement of previously activated $CD4^+$ T cells for B7-mediated co-stimulation is controversial. In some *in vitro* systems, it appears that the requirement of such cells for co-stimulation is less stringent. However, other data suggests that full activation does not occur unless co-stimulation is provided. For example, co-culture of $CD4^+CD45RO^+$ T cells with HLA-mismatched, IFN-γ treated primary epithelial cells (from human thyroid or kidney) has been reported to induce allospecific hyporesponsiveness (Malissen et al. 1988). In the context of an immune response, the purpose of the endothelial cells appears to be to facilitate the entry of activated T cells into the tissue without altering its functional behaviour or activation status (Marelli-Berg et al. 2004). Once in the tissue, antigen-specific T cells could either interact with recruited monocytes and macrophage APCs to be reactivated or, alternatively, with the parenchymal cells to be functionally inactivated. In transplantation, once the donor leukocytes have left the allograft, antigen recognition via parenchymal cells may lead to a form of donor-specific tolerance in T cells previously activated by the direct pathway. This is supported by the reduced frequency of direct pathway reactive T cells with time.

Clearly, as effector cells in the immune response against transplanted organs, T cells play a central role and as such an understanding of T cell phenotypes and diversity is key to developing novel strategies of tolerance induction.

1.1.3.1 T CELL DIFFERENTIATION

Activation of naïve $CD4^+$ T cells results in T cell maturation and differentiation to phenotypes with effector functionality. Individual $CD4^+$ T cell subsets are defined by expression of a combination of specific cytokines, surface markers and transcription factors, and the signalling pathways through which their differentiation is mediated (Atalar et al. 2009). One caveat should be inserted here that accumulating evidence suggests that terminal differentiation of T cells towards a single phenotype may not be absolute and that multi-potency may be retained after differentiation to a given subset. O'Shea *et al*. have provided an up to date article discussing these implications and possible mechanistic insights (O'Shea and Paul 2010). This caveat notwithstanding, both human and murine $CD4^+$ T cells can differentiate from pluripotent naïve cells (Thp) into at least four types of committed helper T cells, namely Th1, Th2, Th17 and regulatory T cells (Tregs) **(Figure 1.2).** Pathways of differentiation and the required signals are very similar between the two species, with only small distinguishing differences (McGeachy and Cua 2008).

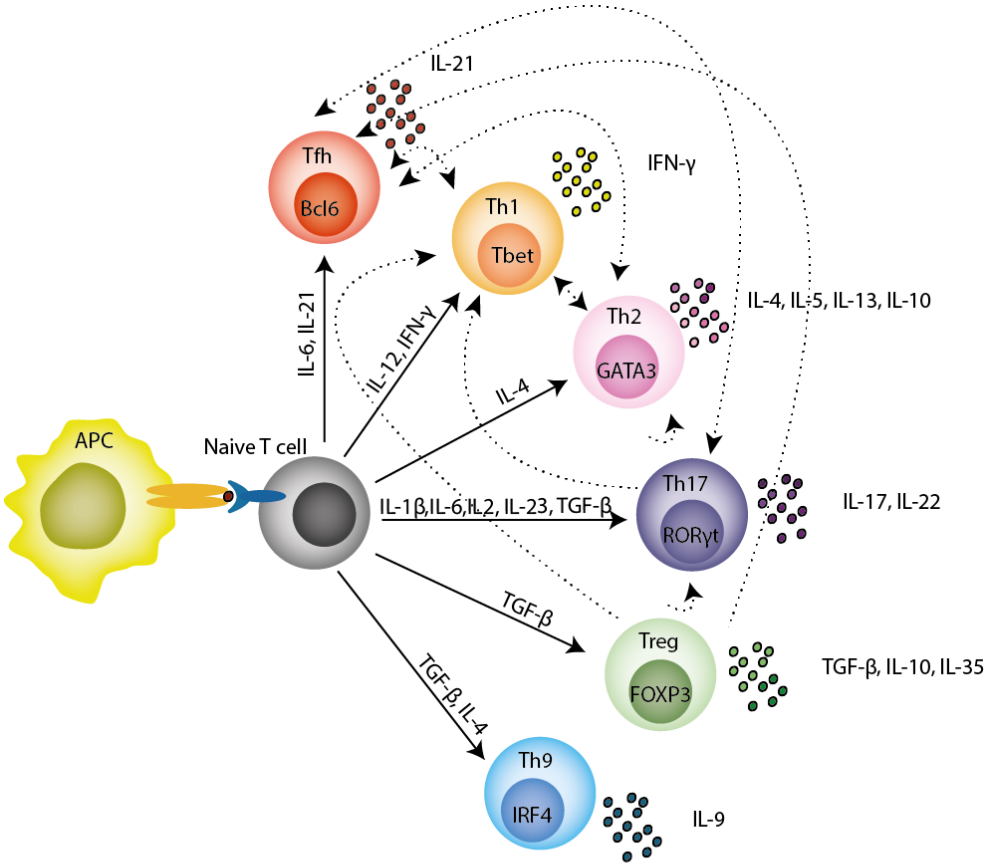


FIGURE 1.2. CD4$^+$ T CELL SUBSET DIFFERENTIATION.

Upon encountering foreign antigens presented by APCs, naïve CD4$^+$ T cells can differentiate into several different T cell subsets, Th1, Th2, Th17, Tregs, and several novel subsets, Tfh and Th9 cells, all heralding distinct immunological roles. These differentiation programs are controlled by different cytokines and each separate CD4 T cell subset can be identified from their lineage specific transcription factors responsible for the regulation and maintenance of their individual functions; T-bet (Th1 cells), GATA3 (Th2 cells), RORγt (Th17 cells), *FOXP3* (Tregs), Bcl6 (Tfh) and IRF4 (Th9). Each subset has its own immunological role *in vivo*: TfH cells produce IL-21 and synergistically interact with B cells in secondary lymphoid tissue. Th1 cells secrete IFNγ, controlling immunity to foreign pathogens. Th2 cells produce various cytokines including: IL-4, IL-5, IL-13, IL-10, which are primarily involved in promoting humoral immunity, protecting against infection. Th17 cells produce predominantly the inflammatory cytokine IL-17 and play an important role in controlling pathogens especially at environmental surfaces. Th9 cells have been shown to be important in protection against helminth infections. Despite the apparent terminal differentiation of all these cells, they cannot be considered to be committed to one cell fate. Lineage plasticity following differentiation is depicted by the dotted arrows. This diagram is far from comprehensive; it is most likely that the future will see various changes and additions to this diagram concerning the differentiation of CD4$^+$ T cells. *Abbreviations: APC- Antigen presenting cells; Bcl6- B cell lymphoma-6; Follicular helper T (Tfh) cells, FOXP3- Forkhead Box P3; IFN- Interferon; IL- Interleukin; IRF-Interferon regulatory factor; RORγt -retinoid related orphan receptor γ; Tbet- T box transcription factor; Tfh- T follicular helper; TGF- β- Transforming growth factor-β; Th- T Helper cell; TNFα- tumour necrosis factor-α; Treg- Regulatory T cell. Adapted from (O'Shea and Paul 2010).*

The evidence for Th1 and Th17 responses in allograft rejection will be reviewed in section 1.1.3.2, whilst regulatory T cells will be discussed in detail in section 1.2.

1.1.3.2. TH1 AND TH17 AND ALLOGRAFT REJECTION

The defining features of Th1 cells are the expression of Interferon (IFN)-γ and differentiation mediated via the signal transducers and activators of transcription (STAT) 1 and STAT4 proteins (Weaver et al. 2007). Th17 cells may be defined by expression of the cytokine interleukin 17 (IL-17) and differentiation through the STAT3 pathway (McGeachy and Cua 2008).

Once differentiated, each lineage is characterised by its own transcription factor - Tbet for Th1 cells (Szabo et al. 2000) and retinoic acid-related orphan receptor (ROR)-α and γT for Th17 cells (Ivanov et al. 2006).

Th1 cells are the classical pro-inflammatory T cell, central to delayed type hypersensitivity responses and critical to the response to intracellular pathogens such as Mycobacteria sp. As such, many studies of transplant rejection have concentrated predominantly on Th1 responses and IFN-γ production. Although this cytokine is undoubtedly elevated in murine models of active transplant rejection, IFN-γ is produced by a number of cell types other than Th1, including natural killer cells (NK) cells, Tregs and $CD8^+$ T cells (Martin-Fontecha et al. 2004, Sawitzki et al. 2005). Therefore, IFN-γ detection does not necessarily denote Th1 activity. Several studies question the role of Th1 cells in allograft rejection (O'Connell et al. 1993, Nickerson et al. 1994) but none so poignantly as rodent heart and kidney transplantation experiments in knockouts of IFN-γ where accelerated graft rejection is invariably the outcome (Miura et al. 2003). Despite the studies outlined above supporting the role of Th1 cells in human transplant rejection, it is important to mention the studies that question this. In paediatric liver transplant patients who tolerated their graft without episodes of rejection, IFN-γ levels remained low, (Gras et al. 2007) however serum IFN-γ concentrations remained significantly elevated at 24 months following renal transplantation in patients without episodes of rejection when compared with healthy controls (Sadeghi et al. 2007). This discrepancy in cytokine levels in tolerant patients questions the role of Th1 cells in rejection. Furthermore, although T-bet, the signature transcription factor of Th1 cells can be identified in renal transplant biopsies during episodes of acute rejection (Hoffmann et al. 2005), in similar fashion to IFN-γ, T-bet is pleiotropic and also expressed by many other cell types including dendritic cells (Alcaide et al. 2007) and NK cells (Townsend et al. 2004) and therefore not specific to Th1 cells during rejection.

Th17 cells produce the highly pro-inflammatory cytokine, IL-17, and are central to the response to extracellular pathogens. These cells have accumulated a wealth of literature as a result of a strong association with autoimmune diseases. Indeed, Th17 cells are now considered principal mediators in the pathogenesis of human autoimmune diseases (Afzali et al. 2007). In the transplant setting, an elevation in IL-17 mRNA in rejecting rat renal allografts and IL-17 protein in infiltrating mononuclear cells can be seen as early as 2 days post transplantation (Loong et al. 2002) while IL-17 protein is elevated in human renal allografts during subclinical rejection together with detectable IL-17 mRNA in urinary sediments (Van Kooten et al. 1998, Loong et al. 2002). An early report of antagonising IL-17 in rat cardiac transplantation concluded significantly prolonged transplant survival yet categorical demonstrations that Th17 cells are capable and sufficient to mediate allograft rejection on their own have only recently been published (Yuan et al. 2008, Yuan et al. 2009). In these models of murine cardiac transplantation, T-bet knockout (devoid of Th1 responses) and T-bet IFN-γ double-knockout recipients of MHC class II-mismatched grafts showed accelerated graft rejection relative to wild-types as a result of profuse tissue infiltration of Th17 cells. In both models, antagonism of IL-17 delayed or prevented graft rejection (Yuan et al. 2008, Yuan et al. 2009).

1.2 Regulatory T cells and Transplantation Tolerance

1.2.1 Mechanisms of Tolerance

Tolerance mechanisms that operate in the thymus before the maturation and circulation of T cells are referred to as 'central tolerance'. Whilst the thymus holds a substantial library of self-antigens, maturing T cells may still hold a degree of autoreactivity, suggesting that central tolerance mechanisms alone are insufficient in entirely educating their development. Additional tolerance mechanisms exist which restrain the number and/or function of T cells slipping through the net of tolerance laid down in the thymus. These mechanisms act on mature circulating T cells and are referred to as 'peripheral tolerance'.

1.2.1.1 Central Tolerance

Circulating bone marrow derived common lymphoid progenitors (CLP) home to the thymus, generating a copious population of thymocytes, which give rise to T lymphocytes (Kondo et al. 1997, Kondo et al. 2000). The thymus is composed of contiguous micro-environmental niches that contain the cues necessary to produce fully mature T cells with functional TCRs (Germain 2002, Takahama 2006). Primordial thymocytes are initially CD4 and CD8 double negative (DN). After T lineage commitment and expansion, TCR gene rearrangement ensues, giving rise to either γδ or αβ progenitors at the CD4 and CD8 DN stage. Successful αβ commitment of the DN cells gives rise to a large number of CD4 and CD8 double positive (DP) thymocytes with somatic recombination of TCR genes resulting in a

broad repertoire of distinct αβ TCR of random specificity. The TCR affinity for self-peptide MHC determines a thymocyte's fate from this point (Klein et al. 2014) **(Figure 1.3).**

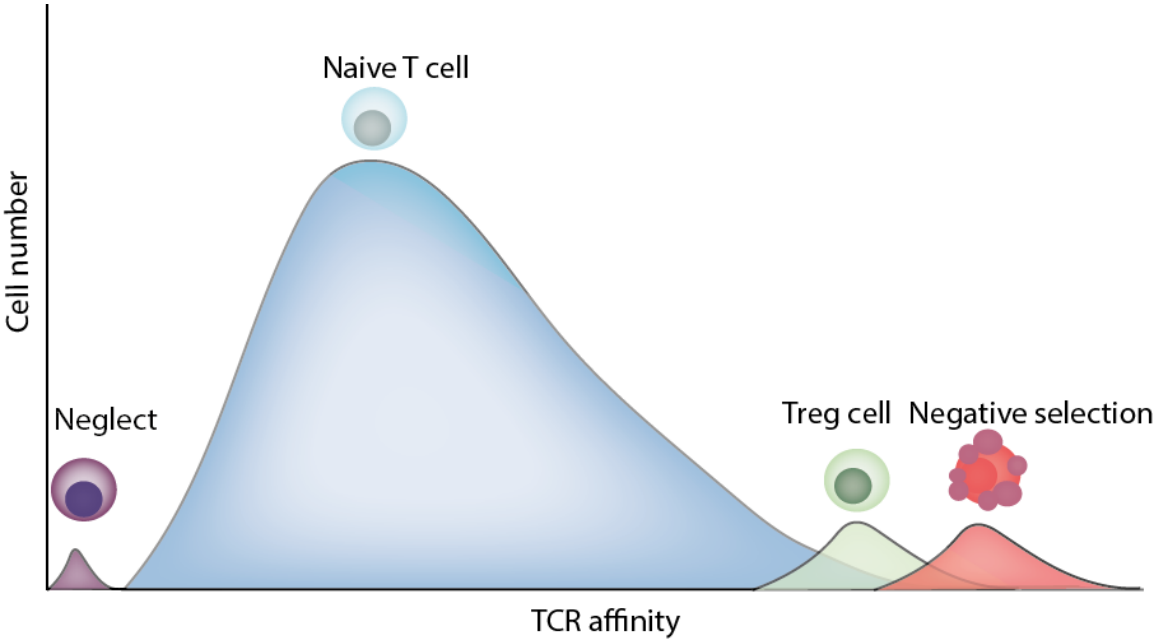


FIGURE 1.3. THYMOCYTE SELECTION AFFINITY MODEL.

The strength of the interaction between T cell receptors, expressed on thymocytes, and self-peptide MHC determines the fate of these cells. Positive selection of thymocytes occurs following weak- intermediate interactions with self-MHC whereas those which have zero affinity for self-MHC die by neglect. Thymocytes with a high affinity for self-MHC are subject to negative selection. Clonal diversion, redirecting cells to take up an immunoregulatory lineage (Tregs), and deletion are the two main forms of negative selection. Clonal diversion is said to occur at an optimal affinity where positive selection and deletion sit either side. In addition, thymocytes that are negatively selected may be subject to receptor editing and/or anergy in order to avert any possibility of autoimmunity. *Abbreviations: TCR- T cell receptor; Treg- regulatory T cell. Adapted from (Klein et al. 2014).*

DP thymocytes expressing TCRs that do not bind self-peptide MHC complexes die by neglect. Those with a low affinity for self-peptide MHC complexes are positively selected to differentiate into CD4 or CD8 single positive (SP) thymocytes. However, thymocytes with high affinity for self peptide-MHC undergo negative selection, thus preventing the development of autoimmunity. Following negative selection cells are subject to revisory alterations in order to avert autoimmunity.

Receptor editing, is a process whereby the thymocytes with high affinity for self-peptide MHC are instructed to generate a second rearrangement of the TCR α loci, thereby altering the specificity of the TCR (Wang et al. 1998, McGargill et al. 2000, Santori et al. 2002, Mayerova and Hogquist 2004).

Anergy, or a state of induced unresponsiveness has also been implicated (Hammerling et al. 1991). Although, the relative contribution of the receptor editing and anergy to central tolerance is thought to be minimal, with anergy described most commonly as a tolerance mechanism operating in the periphery.

Instead, clonal diversion and deletion of high affinity thymocytes determines their predominant fate. The former describes the diversion of high affinity thymocytes into lineages that attain immunoregulatory function, whilst the latter involves the induction of apoptosis in self-reactive clones. Of note, clonal deletion is the predominate mechanism by which central tolerance is achieved (Liston and Rudensky 2007) (Baldwin et al. 2004, MacDonald and Mycko 2007).

1.2.1.2 Peripheral Tolerance

Peripheral tolerance mechanisms exist, namely deletion of self-reactive T cells, anergy and regulation, to ensure the continuation of tolerance when lymphocytes first encounter their cognate self-antigens outside of the thymus.

1.2.1.2.1. Peripheral Deletion

Clonal deletion of autoreactive T cells has been shown to occur through apoptosis via activation of the Fas/FasL pathway and the Bim dependent mitochondrial pathway (Mueller 2010). In agreement, Bim deficient and Fas deficient (lpr/lpr) mice display defects in peripheral tolerance, leading to lymphadenopathy, splenomegaly and the development of autoimmunity suggesting the involvement of both molecules in the peripheral deletion of T cells (Hutcheson et al. 2008, Weant et al. 2008).

1.2.1.2.2. T cell anergy and costimulatory signals

T cell activation primarily involves the TCR signal. However, T cells are not able to mount an immune response without a second costimulatory signal. This signal involves the interaction of the CD28 receptor on T cells with two ligands, B7.1 (CD80) ad B7.2 (CD86), expressed on APCs, promoting T cell activation, proliferation and survival, after T cell interaction with APC **(Figure 1.4).** In parallel activation of T cells in the absence of CD28 has been shown to result in an anergic state (Bour-Jordan et al. 2011), with animal models reporting that inhibition of the CD28/B7 interaction results in transplantation tolerance (Li et al. 1999). A concept already discussed in section 1.1.3 concerning the direct pathway of presentation through epithelial cells.

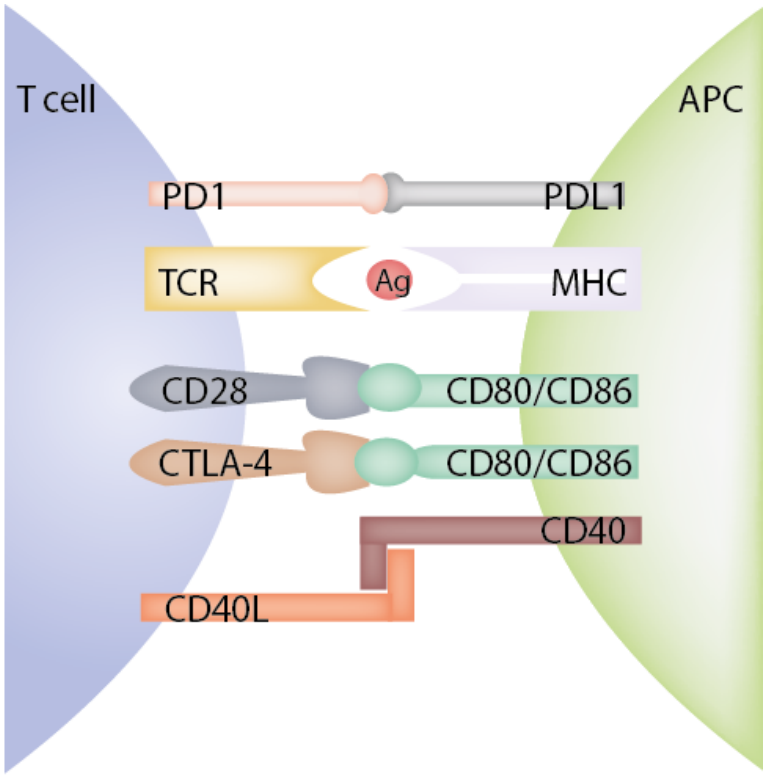


FIGURE 1.4. T CELL ACTIVATION

Interaction between naïve T cells and APCs initiates T cell activation. Stimulation begins following contact of the TCR with antigen-bound MHC. This interaction is considered as signal 1. However, for complete activation of the T cell, additional costimulatory signals are required. Primarily, the interaction between CD28 and CD80/86, expressed on the T cell and APC respectively, is the most important co-stimulatory signal, identified as signal 2. Various other additional signals are also thought to be involved in T cell activation including CD40/CD40L ligation. The interaction between CTLA-4 and CD80/86 has been shown to inhibit T cell activation by out competing CD28 for its ligand on APCs, thus preventing signal 2 from propagating. *Abbreviations: Ag- antigen; APC- antigen presenting cell; CD- cluster differentiation; CTLA-4-cytotoxic T-lymphocyte-associated protein 4; L- ligand; MHC- major histocompatibility complex; PD-Programmed death; PDL-1- Programmed Death Ligand; TCR- T-cell receptor. Adapted from (Alpdogan and van den Brink 2012).*

Autoreactive T cells also express a number of immunosuppressive molecules including programmed dealth-1 (PD-1), CD5 (Hawiger et al. 2004), T cell immunoglobulin and mucin domain-3 (TIM-3) (Anderson and Anderson 2006) and cytotoxic T lymphocyte antigen-4 (CTLA-4) (Eggena et al. 2004, Mallone et al. 2005) (mentioned in detail in section 1.2.4.1).

Consequently autoreactive T cells with relative low avidity for their cognate antigen have a high threshold for activation and are functionally anergic (Mallone et al. 2005). In contrast those with a high avidity are particularly sensitive to Fas and Bim dependent apoptosis, therefore, these potentially pathogenic cells are quickly removed from the T cell pool (Marrack and Kappler 2004) Of note, also within the periphery, autoreactive T cells are constrained, by a group of regulatory immune cells.

1.2.1.2.3. SUPPRESSION OF IMMUNE RESPONSES; REGULATORY CELLS

The proposal of the existence of a distinct subset of T cells able to suppress immune responses was first put forward in the 1970s which led to extensive research in order to identify these 'suppressor T cells. (Gershon and Kondo 1970). It was not till the mid 90's when, a thymic derived lymphocytic population, coined Tregs, were first defined. These cells play a major role in the development of tolerance by suppression of immune responses (Wing and Sakaguchi 2010).

The past two decades have seen the discovery of many different types of regulatory cells including; $CD8^+$ T cells (Reibke et al. 2006), $CD4^-CD8^-$ double negative T cells (Zhang et al. 2000), $CD8^+CD28^-$ (Haribhai et al. 2007), NK T cells (Monteiro et al. 2010), and γδ T cells (Hayday and Tigelaar 2003), but these are less well studied compared to their $CD4^+$ regulatory T cells counterparts, further characterised by high

and stable expression of surface interleukin-2 receptor α chain (IL-2Rα, $CD25^{hi}$) (Sakaguchi et al. 1995). This thesis is based primarily on $CD4^{+}CD25^{+}$ T regulatory cells (Tregs).

1.2.2 Regulatory T cell Development; Thymic and Peripheral Origins of Regulatory T cells

Tregs are divided into two main subsets, based on their site of differentiation, namely thymus derived natural Tregs (tTregs) and peripherally induced Tregs (pTregs), (Fehervari and Sakaguchi 2004). tTregs are spawned from negatively selected thymocytes, whereas the conditions favoring the generation of pTregs include suboptimal DC activation, sub-immunogenic doses of agonist peptide, mucosal administration of peptide and antigenic encounter in the presence of environmental cytokines: transforming growth factor-β (TGF-β) , IL-2 and retinoic acid (Povoleri et al. 2013).

There are at least two well defined populations of pTregs; Th3, first identified from their role in oral tolerance through the secretion of TGF-β (Chen et al. 1994) and Tr1, characterised on the basis of their role in preventing autoimmune colitis (Groux et al. 1997) and their ability to secrete large amounts of IL-10 (Levings et al. 2001, Barrat et al. 2002). As such pTregs are implicated in the induction of oral and gut tolerance (Coombes et al. 2007) and generated in chronically inflamed and transplanted tissues (Cobbold 2008).

Of note, the functional distinction of thymic and peripherally derived Tregs has not been clearly established, posing challenges in classifying the definitive proportions of these two subsets in secondary lymphoid organs and non-lymphoid tissues alike. Despite great interest in identifying surface markers that differentiate these Tregs, no marker has been conclusively identified to date. In mouse, neuropilin (Nrp-1) expressed on tTregs can differentiate these cells from their peripherally derived counterparts, which do not express this molecule (Weiss et al. 2012, Yadav et al. 2012). However, this distinction does not hold true for human Tregs.

Although, it should be noted that other than their different sites of differentiation, Tregs, particularly those in humans, are highly heterogeneous. Various different surface and intracellular immunological markers have been studied, defining Tregs based on their functional characteristics, migration and lineage plasticity, detailed below.

1.2.3 Regulatory T cell markers

CD25

The IL-2 receptor α chain, CD25, is undoubtedly the most useful marker for Treg identification and isolation in view of its extracellular expression. Despite its transient presence on activated effector T cells (Sakaguchi et al. 1995, Kmieciak et al. 2009) a high constitutive expression of CD25 is commonly used to define and isolate functionally suppressive Tregs for research purposes (Suri-Payer et al. 1998).

The integral expression of CD25 introduces the critical role of IL-2 in the development and survival of Tregs (Bensinger et al. 2004, Fontenot et al. 2005). In this regard, IL-2 neutralisation (Setoguchi et al. 2005) and deficiency (Kramer et al. 1995) has been shown to result in Treg defects and ultimately the onset of autoimmunity (O'Shea et al. 2002).

FOXP3

Further characterisation and understanding of Treg cell biology came from the discovery of FOXP3, an intracellular transcription factor known to play a crucial role in the development and function of Tregs in a highly specific manner (Hori et al. 2003). Rare mutations of the *FOXP3* gene have been linked with the development of immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX), leading to organ-specific autoimmune diseases including insulin-dependent diabetes mellitus and various hematological disorders (Hori et al. 2003). Furthermore, the importance of FOXP3 in the safeguarding of Treg phenotype and function has been reiterated in studies where a loss/diminution of FOXP3 expression in Tregs has been shown to affect the competency of these cells acquiring certain effector T cell properties, including production of cytokines such as IL-2, IL-4, IL-17 and IFN-γ (Wan and Flavell 2007). Described in detail in section 1.2.5.

Additionally, while *FOXP3* has been termed a ‘master control gene’, specifically with regards to Treg development, its expression is not uniformly homogenous. In contrast to mice, where *foxp3* is expressed purely on Tregs, in humans, increasing evidence

has shown that effector cells can transiently express FOXP3, with no associated regulatory activity. Based on such studies and taking also into account its intracellular expression, this marker in isolation cannot be considered to be entirely sufficient in demarcating Tregs (Huehn et al. 2009).

CD127

Tregs are typically described as $CD127^{Lo}$, based on reports that expression of the α chain of the IL7 receptor, CD127, inversely correlates with FOXP3 expression and functional suppressive capabilities (Liu et al. 2006). As such, the combination of CD25, FOXP3 and CD127 are considered to be the most stringent markers in defining Tregs in the research setting.

CD45RA

The classification of human Tregs into both tTregs and pTreg subsets infers that these cells cannot be considered to be functionally homogeneous. Following the recent discovery of naïve suppressive $FOXP3^{+}$ cells ($CD45RA^{+}$) present in the cord blood and in adult blood, and $FOXP3^{+}$ cells which express a memory-like phenotype ($CD45RA^{-}$), it has been proposed that three phenotypically and functionally distinct sub-populations based on the differential expression of CD25, FOXP3 and CD45RA can be defined: Population I ($CD25^{++}FOXP3^{+}CD45RA^{+}$) classified as resting Tregs, population II ($CD25^{+++}FOXP3^{hi}CD45RA^{-}$) termed activated Tregs and population III ($CD25^{++}FOXP3^{+}CD45RA^{-}$) which was proposed to consist of non-suppressive $FOXP3^{Lo}$ cells (Miyara et al. 2009). Further analysis of the three populations by

Miyara et al. revealed that population I and II were both able to suppress *in vitro* with population II displaying a higher expression of CTLA-4, suggesting a more prevalent role in cell-mediated immunological suppression (described in detail in section 1.2.4.1). Population III however was shown to be non-suppressive (Miyara et al. 2009).

Demarcation of these three populations was also able to depict the differentiation dynamics of $FOXP3^+$ Tregs. Resting Tregs, following stimulation *in vivo* upregulate their FOXP3 expression and mature to terminally differentiated activated Tregs maintaining its apoptotic pool. Miyara et al. suggested that population III had the greatest potential to differentiate into inflammatory Th17 cells, inferred from their relative IL-17 production following cytokine stimulation (Treg plasticity is discussed in section 1.2.5). The three comparative populations are found in different proportions in certain biological environments and their analysis can prove to be instrumental in identifying the immunological pathophysiology of disease.

It should, however, also be noted that the functional characteristics of population III are debated. Booth et al. and data from my laboratory indicated that both $CD45RO^+$ and $CD45RA^+$ Treg subsets are equally suppressive, population III representing a bona fide Treg subpopulation, bearing T cell memory markers (Booth et al. 2010).

HLA-DR

The human MHC can be divided into two distinct subsets of human leukocyte antigen (HLA) class I (HLA-A,-B and–C) and class II (HLA-DP,-DQ and–DR) and is known to play a central role in the presentation of peptide to T cells. The expression of

MHC-II DR has primarily been seen as a marker of 'activated' T cells. However, the two subsets, HLA-DR$^+$ and HLA-DR$^-$, have been used to define two functionally distinct Treg subsets. Baecher-Allan et al. defined the HLA-DR$^+$ (MHCII) Treg subset as a functionally mature subpopulation associated with early-contact dependent suppression, however, contact mediated suppression was not constrained by the class II dimer (Baecher-Allan et al. 2006). In a follow up study, Beriou et al. found that HLA-DR$^-$ Tregs, although retaining their suppressive function, produced IL-17 in an inflammatory setting (Beriou et al. 2009).

CD27

The co-stimulatory molecule CD27 is member of the TNF receptor superfamily, known to bind its ligand CD70. This interaction is important in the co-stimulation of T cells. CD27 is initially upregulated following TCR engagement, however, following repeated antigenic stimulation, levels drop (Hintzen et al. 1995). As a result, it has been proposed that CD27 can be used as a marker of stable Tregs since cells expressing CD27^{Lo} may represent a subset of previously activated cells (De Jong et al. 1992). Furthermore, the co-expression of CD27 and CD62L, a marker known to be involved in Treg lymph node homing, has been used to identify a population of Tregs with high suppressive function *(Scotta et al. 2013)* (*Bluestone et al. unpublished data).*

CXCR3

In homeostatic conditions Tregs suppress effector T cell activation in lymphoid tissue. However, it has been reported that following a state of chronic inflammation, Tregs are found at the inflammatory site where they are able to directly suppress immune responses. In order to migrate from secondary lymphoid tissue to peripheral sites, Tregs must down regulate their relative expression of CCR7 and CD62L (Oo et al. 2010) that are known to be specific lymphoid homing molecules. The upregulation of various specific chemokine receptors also aids the positioning and recruitment of Tregs to chronically inflamed tissue. The chemokine receptor CXCR3, has recently been proposed to be expressed on a subset of liver-infiltrating Tregs allowing transendothelial migration across the hepatic sinusoids, which was further supported by the observation of increased numbers of CXCR3 ligands, CXCL9, 10 and 11 in chronic inflammatory liver disease (Shields et al. 1999).

1.2.4 Mechanism of suppression

In vitro studies have demonstrated that the immunosuppressive qualities native to Tregs manifest through a variety of mechanisms **(Figure 1.5)**, namely; modulation of APC maturation and function, anti inflammatory cytokine production, induction of apoptosis in target cells and disruption of metabolic pathways.

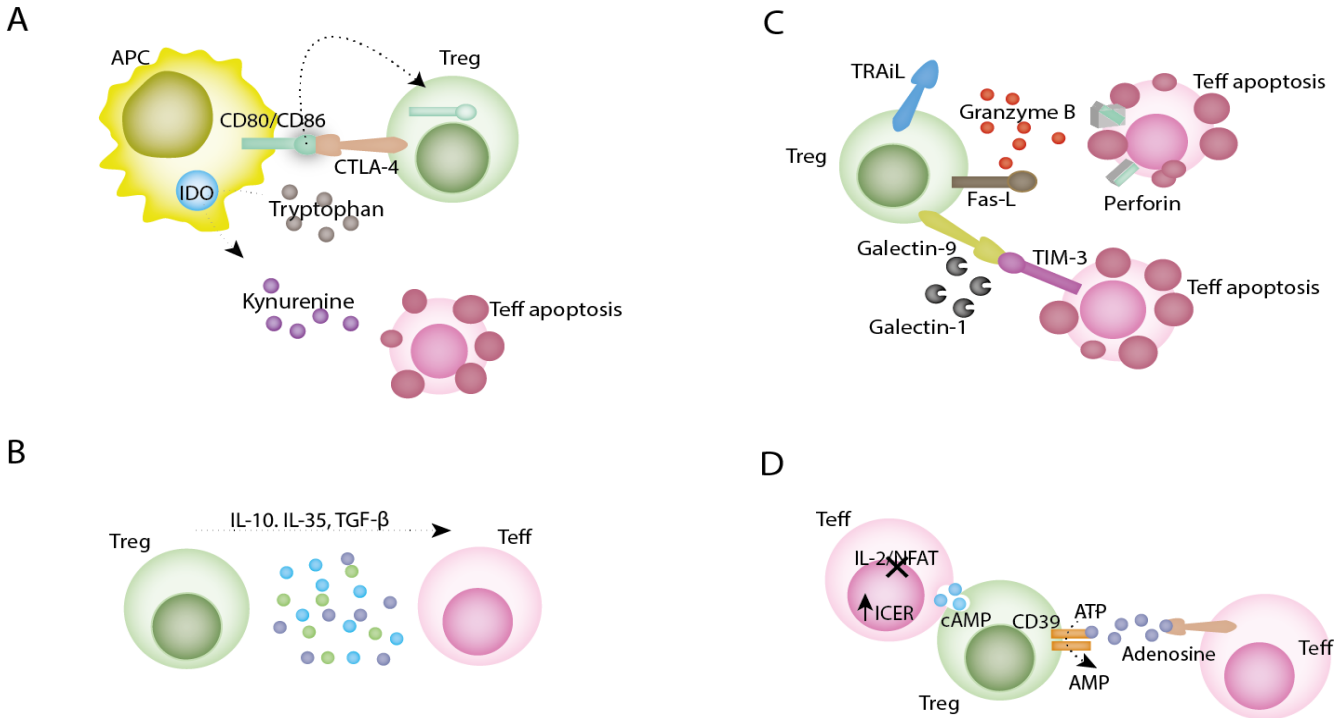


FIGURE 1.5. MECHANISMS OF REGULATORY T CELL SUPPRESSION

A. Modulation of APC maturation and function. The interaction of CTLA-4 on Tregs with its ligand CD80/86 on APCs, delivers a negative signal for T cell activation. CTLA-4's mechanism of action is varied including: the capture of its APC expressed ligands and subsequent trans-endocytosis and also the upregulation of IDO and the generation of kynurenines. **B. Anti-inflammatory cytokine production.** The secretion of anti-inflammatory cytokines such as: IL-10, IL-35 and TGF- β has been linked with inhibition of T cell activation *in vivo* **C. Induction of apoptosis.** Tregs have the capacity to directly induce apoptosis via granzyme A/B and perforin, TRAIL, the Fas/Fas-ligand pathway, the galectin-9/TIM-3 pathway, or the production of galectin-1. **D. Disruption of metabolic pathways.** The ectoenzymes CD39 and CD73, expressed on Tregs, result in the metabolism of ATP to AMP and in turn producing the immunoregulatory purine, adenosine. Tregs have also been found to express high levels of intracellular cAMP. This is transferred to Teffector cells through gap junctions which leads to the upregulation of ICER and in turn the inhibition NFAT and Il-2 transcription leading to apoptosis by IL-2 deprivation. *Abbreviations: APC- Antigen presenting cell; AMP- Adenosine monophosphate; ATP- Adenosine triphosphate; cAMP- cyclic adenosine monophosphate; CD- Cluster differentiation; CTLA-4-cytotoxic T lymphocyte antigen-4;* DC- dendritic cells*; ICER- inducible cAMP early repressor, IDO- indoleamine 2,3-dioxygenase IL- interleukin; NFAT- nuclear factor of activated T cells; TGF β -transforming growth factor-β, TIM-3- T cell immunoglobulin and mucin domain-3; TRAIL- tumour necrosis factor-related apoptosis-inducing ligand; Treg- Regulatory T cells. Adapted from (Vignali et al. 2008).*

1.2.4.1. MODULATION OF APC MATURATION AND FUNCTION

The expression of CTLA-4 on both murine and human Tregs (Read et al. 2000, Takahashi et al. 2000, Qureshi et al. 2011) has been linked with Treg mediated immune suppression. In this regard, it has been demonstrated that CTLA-4 deficiency or blockade in mice results in spontaneous autoimmunity, which can be ameliorated by Tregs (Bachmann et al. 1999, Takahashi et al. 2000). Further supporting studies have reported that CTLA-4 blockade abrogates the protective effects of Tregs in murine colitis models (Read et al. 2000). However, there is contradictory evidence surrounding the definitive weight of CTLA-4 in Treg immunosuppression following reports that CTLA-4 deficient Tregs could still suppress through the compensatory mechanisms involving TGF-β and IL-10 *in vitro* and *in vivo* (Tang et al. 2004, Read et al. 2006). Similarly, Baecher-Allan et al. did not find an involvement of CTLA-4 in Treg mediated suppression of Teffector proliferation (Baecher-Allan et al. 2001, Levings et al. 2001).

Despite these studies, the most recognised molecular mechanism behind Treg mediated suppression through CTLA-4 is centered on the disruption of the co-stimulatory signal (Cederbom et al. 2000). Mechanistically, downregulation of CD80/CD86 on target APCs by CTLA-4 expressing Tregs involves the 'capture' of these ligands by CTLA-4, a process known as trans-endocytosis (Qureshi et al. 2011), thereby indirectly inhibiting T effector activation by APCs *in vitro* (Oderup et al. 2006) and *in vivo* (Wing et al. 2008).

However, other proposed mechanisms of action of CTLA-4 have included the upregulation of IDO (indoleamine 2, 3-dioxygenase) expression in DCs, an enzyme involved in the degradation of tryptophan to kynurenine, in turn starving effector T cells of their essential sustenants and directing cell cycle arrest (Mellor and Munn 2004).

1.2.4.2. Suppression via anti-inflammatory cytokine production; TGF-b, IL-10 and IL-35

Whilst *in vitro* studies have highlighted the importance of cell-to-cell contact in the materialization of Treg suppression (Shevach et al. 2001, Kullberg et al. 2005), *in vivo* models have also stressed the involvement of anti-inflammatory cytokines, TGB-β, (Powrie et al. 1996, Fahlen et al. 2005) IL-10 (Hara et al. 2001) and IL-35 (Collison et al. 2007) in Treg mediated suppression.

In this regard, it has been shown that Tregs can produce high amounts of membrane-bound and soluble forms of TGF-β with *in vitro* studies confirming the role of this cytokine in suppression of T cell proliferation (Nakamura et al. 2001, Levings et al. 2002) and its production by Tregs necessary to prevent colitis (Read et al. 2000). In the same setting, Asseman et al. provided evidence for the role of IL-10 in Treg mediated suppression, whereby blocking of IL-10 or using IL-10 deficient Tregs abolished the protective effects of Tregs in colitis (Asseman et al. 1999).

IL-35 is a recently discovered cytokine implicated in Treg mediated suppression and has been shown to directly inhibit T-effector proliferation (Collison et al. 2007). Its precise role, however, is yet to be determined.

1.2.4.3. SUPPRESSION BY INDUCTION OF APOPTOSIS

Human Tregs expressing the serine protease granzyme A have been reported to kill $CD4^+$ T cells and other target cells in a perforin-dependent cytotoxic manner (Grossman et al. 2004). In another report contact dependent suppression of T-effector proliferation by activated murine Tregs *in vitro* was described to be partially granzyme B dependent; however in contrast to the results of Grossman et al., perforin was not involved (Gondek et al. 2005).

In line with this, studies have further shown that Tregs express the death ligands: tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) (Ren et al. 2007) and Fas-ligand (Strauss et al. 2009), which upon binding of the death receptors expressed by their target, induce the formation of the death domain, in turn endorsing the initiation of the caspase cascade (Dubin and Kolls 2008).

Wang et al. also showed that Tregs expressed galectin-9, which binds to TIM-3, expressed by activated effector T cells, leading to intracellular calcium influx and the activation of the calpain caspase-1 pathway (Wang et al. 2009). The expression of galectin-1 on Tregs has also been demonstrated (Garin et al. 2007), but the mechanism by which galectin-1^+ Tregs induce T cell apoptosis is not fully understood (Kovacs-Solyom et al. 2010).

1.2.4.4. SUPPRESSION BY DISRUPTION OF METABOLIC PATHWAYS

Extracellular adenosine triphosphate (ATP) serves as an indicator of tissue damage in the immune system. It can be released from cells following an inflammatory response

and binds to purinergic receptors (P2) which are highly expressed on immune and epithelial cells, potentiating a pro-inflammatory response with the secretion of inflammatory cytokines including IL-1β (Imai et al. 2000). Kobie et al. showed that Tregs express high levels of the cell surface enzyme ecto-5-nucleotide CD73 (Kobie et al. 2006) and that this molecule converts extracellular adenosine-5-monophosphate (AMP) to adenosine, which is known to have immunosuppressive and anti-proliferative effects (Robson et al. 2006). Of note, however, the ectonucleotide triphosphate diphosphohydrolase-1, CD39, also found on Tregs, provides the substrate for CD73, hydrolyzing extracellular adenosine-5-triphosphate (ATP) into the nucleotide AMP (Deaglio et al. 2007).

$CD4^{+}CD25^{hi}CD39^{+}$, predominantly expressing FOXP3, were also found to suppress the release of the pro-inflammatory cytokine IL-17 alongside IFN-γ and IL-2 from Th17 cells while $CD4^{+}CD25^{hi}CD39^{-}$ T cells had an increased propensity to produce IL-17 (Fletcher et al. 2009). Furthermore, the reconstitution of positively selected CD39-null mouse cells with apyrase (involved in the catabolism of ATP) reversed their increased susceptibility to develop auto-immune diseases and a Th-1 skewed immune response (Dwyer et al. 2007).

Moreover, studies have proposed that Tregs express high levels of intracellular cyclic adenosine monophosphate (cAMP) with implications in Treg suppressive ability (Bopp et al. 2007). Bopp et al, proposed that cAMP is an important component of Treg mediated suppression since suppression of IL-2 transcription and proliferation could be partly abrogated by a cAMP antagonist or a gap junction inhibitor (Bopp et al. 2007). In this regard, Fassbender et al. showed that the induction of cAMP in

murine DCs upon Treg co-culture contributed to the suppression of DCs (Fassbender et al. 2010). Bodor et al. further proposed that cAMP can be directly transferred to effector T cells via gap junctions, leading to the upregulation of the inducible cAMP early repressor (ICER) (Bodor et al. 2000), which limits nuclear factor activated T cells (NFAT) and IL-2 transcription (Bodor et al. 2012).

1.2.4.5. THE ROLE OF EXOSOSOMES IN REGULATORY T CELL MEDIATED IMMUNE SUPPRESSION

Exosomes are lipid-bound nanovesicles formed by the inward budding of endosomal membranes (Cho et al. 2009). It has been found that many cells can secrete exosomes, such as tumor cells, DCs, lymphoid cells, mesothelial cells, epithelial cells, and cells from different tissues or organs (Admyre et al. 2007). Although their precise function *in vivo* is still unknown, exosomes have been proposed to play a role in antigen presentation, immunoregulation, and signal transduction (Chaput et al. 2004). Some studies have supported the view that exosomes contribute to the induction and maintenance of transplantation tolerance. In this regard, in a rat model of allogeneic heart transplantation, intravenous inoculation of DC cell-derived exososmes resulted in a delay of acute allograft rejection and the induction of a significant propagation of allograft survival (Peche et al. 2003). Interestingly, it has also been proposed that Tregs release exosomes and this is one mechanism by which they modulate immune responses. In agreement, a recent study, using a rat model of kidney transplantation, Yu et al. showed that autologous Treg derived exososmes postponed allograft rejection and prolonged the survival of transplanted kidney (Yu et al. 2013). From their *in vitro* analysis of the function of the exososmes, they further concluded that the exososmes possessed the capacity to suppress T cell proliferation. Of note, recently my laboratory have further supported that Treg derived exosomes display immune-modulating properties *in vitro,* which is attributed to the production of adenosine by CD73 detected on the exosomes (Smyth et al. 2013).

1.2.5 Stability and plasticity of regulatory T cells

There is now growing evidence that Tregs are plastic with a potential to convert into proinflammatory cells (Zhou et al. 2009). In agreement, the presence of a population of $FOXP3^+$ T cells capable of secreting the pro-inflammatory cytokine, IL-17, has been observed in human peripheral blood, thus questioning the stability of these cells.

In this regard, the importance of assessing Treg stability has been proposed, especially in view of the clinical application of these cells.

As eluded to in section 1.2.3 the stability and function of Tregs has been shown to depend on high expression of FOXP3, which is controlled at an epigenetic and protein level. It has been demonstrated that between the *FOXP3* promoter and the first exon lies a stretch of highly conserved, non coding sequence, that is differentially methylated in tTregs, pTregs and Teffectors (Baron et al. 2007, Polansky et al. 2010). This sequence, referred to as the Treg-specific demethylated region (TSDR) is crucial at maintaining high FOXP3 expression in Tregs (Zheng et al. 2010). Mechanistically, it has been proposed that the demethylated TSDR region allows binding of transcription factor complex that contains CREB/ATF, STA5, ETS-1 and *FOXP3* itself to maintain transcriptional activity (Baron et al. 2007, Polansky et al. 2010).

In addition to this epigenetic mechanism in controlling FOXP3 expression, FOXP3 function and expression is also controlled at the post translational level by acetylation of its many lysine residues (Wang et al. 2009, Xiao et al. 2010) Of note, it has been demonstrated that the acetylation of lysine residues in FOXP3 prevents their polyubiquination and subsequent degradation by proteasomes (van Loosdregt et al. 2011), promoting subsequent DNA transcription. Such studies merely highlight that

the stability of FOXP3 expression in Tregs is controlled at both an epigenetic and translational level to ensure the stability of the lineage.

Despite this strict government of FOXP3 expression, emerging data suggests that Tregs can downregulate FOXP3 in the presence of inflammatory cytokines. In agreement, Yang et al., have shown that exposure of Tregs to IL-6 and IL-1 *in vitro* results in the expression of IL-17 (Yang et al. 2008). *In vivo*, loss of FOXP3 has also been documented in the setting of autoimmune disease (Zhou et al. 2009) fetal acute infections (Oldenhove et al. 2009), TLR stimulation (Sharma et al. 2010) and homeostatic proliferation (Komatsu et al. 2009). Moreover, the study by Hoffman et al. further concluded that upon repeated antiCD3/CD28 stimulation in culture, Tregs parted with their constitutive expression of FOXP3, in turn forfeiting their stability (Hoffmann et al. 2009).

As such, to determine the origin of the unstable Tregs, the extrinsic factors resulting in their instability and the fate of these 'exTregs' is of particular importance.

In this regard, Hori et al. proposed a 'heterogeneity model', suggesting that FOXP3 expression does not necessarily determine Treg lineage commitment. They showed that uncommitted FOXP3 positive cells can lose FOXP3 expression, acquiring transient activation-induced FOXP3 expression; converting to effector like ex-FOXP3 cells under inflammatory conditions, or committing to a Treg fate (upon demethylation of TSDR) (Hori 2011). Consistent with this, epigenetic analysis shows that tTregs and pTregs have partially methylated TSDR, despite high levels of FOXP3 expression (Floess et al. 2007, Wieczorek et al. 2009) with a proposal that lack of

further commitment cues and/ or exposure to inflammatory cytokines and/or IL-2 deprivation at this developmental stage may cause these cells to abort Treg development program, lose suppressive function and take on an effector phenotype.

Of note, however, it is important to make the distinction between Treg instability and functional specialisation. Studies have also shown that whilst under inflammatory conditions Tregs can acquire the ability to produce effector cytokines while still maintaining high FOXP3 expression and suppressive activity. In support of this, it has been demonstrated that IL-17 is produced by a subset of highly suppressive human Tregs that express CCR6, the chemokine receptor used by Th17 cells for their recruitment to sites of inflammation (Voo et al. 2009).

Moreover, our group and others, have shown the expression of CD161, the killer cell lectin-like receptor subfamily B, to be expressed on a subpopulation of human Tregs, that produce IL-17 upon *in vitro* activation in the presence of IL-1β, but not IL-6. In addition, evidence has also supported the suppressive capacity of these cells (Afzali et al. 2013, Pesenacker et al. 2013).

These studies highlight that Treg expression of effector cytokines alone cannot be simply viewed as a marker of plasticity or lack of stability, but may be a hallmark of their functional specialization. In this support, Campbell and Koch et al. have shown that Treg expression of transcription factors and cytokine specific for Th1, Th2 and Th17 enables them to control inflammation mediated by Th1, Th2 and Th17 cells, by responding to the same environmental cues (Campbell and Koch 2011).

1.2.6 Regulatory T cells in Transplantation; adoptive transfer and lessons learnt from pre-clinical data

The term 'adoptive immunity' was first coined in 1954 by Billingham et al. (Billingham et al. 1954), who were able to show that passive transfer of primed immune cells can generate immunity in the recipient. Subsequently, numerous animal studies have demonstrated the effectiveness of this adoptive transfer of immunity in both cancer and infectious disease (Dudley and Rosenberg 2003).

Moreover, in humans the isolation and manufacture of IL-2 permitted, for the first time, the *ex vivo* culture and expansion of T cells (Lotze et al. 1980)

In addition, many transplant researchers found that $CD4^+$ T cells were responsible for donor specific tolerance and it was the study by Hall et al. which concluded that transplant tolerance was mediated by $CD4^+CD25^+$ cells. In this study they showed that in cyclosporin-treated rats with long-term cardiac allograft survival, the adoptive transfer of $CD4^+CD25^+$ T cells resulted in tolerance (Hall et al. 1990).

The application of Tregs in the context of organ transplantation was further supported by the seminal work by Sakaguchi et al. demonstrating that Tregs from naive mice prevented rejection of allogeneic skin grafts in T cell deficient nude mice given $CD25^-$ T cells (Sakaguchi et al. 1995).

Subsequently, a series of pre-clinical rodent models of skin and cardiac transplantation demonstrated that Tregs present in the recipient at the time of transplantation are critical in the induction and maintenance of tolerance (reviewed in

(Wood and Sakaguchi 2003). Additionally, mouse models of bone marrow transplantation (BMT) further supported the importance of adoptive Treg therapy, whereby the transfer of freshly isolated Tregs together with the bone-marrow allograft resulted in amelioration of GVHD and facilitated engraftment (Joffre et al. 2004, Hanash and Levy 2005).

Moreover, adoptive transfer of Tregs has been shown to prevent rejection in other murine models of transplantation, such as pancreatic islets (Sanchez-Fueyo et al. 2002).

1.2.6.1. Antigen Specific regulatory T cells and Transplantation

An issue for consideration in Treg cell therapy is the relevance of Treg allospecificity with the significant advantage that the immunomodulatory function of these cells would be concentrated at the site of alloantigen and immune activation (Dijke et al. 2008). An additional advantage of alloantigen-specific cellular therapy is that undesirable pan-suppression, resulting in increased risk of infections and cancers, is less likely to occur.

Although, the indirect pathway has been implicated in acute graft rejection (Auchincloss et al. 1993) this pathway may be the major driver of chronic allograft rejection (Wise et al. 1998). Indeed much experimental and clinical evidence suggests that for tolerance to occur this is the pathway that needs to be regulated. It is clear from clinical studies that regulatory T cells contribute little, if at all, to direct pathway hyporesponsiveness (Game et al. 2003) and in contrast depleting Tregs reveals

significant indirect pathway anti-donor alloresponses in stable renal transplant patients (Salama et al. 2003). In addition, studies in mice and humans show that the indirect pathway of allorecognition is used by Tregs for immunoregulation (Wise et al. 1998, Hara et al. 2001, Yamada et al. 2001, Quezada et al. 2003, Spadafora-Ferreira et al. 2007).

Thus, in the induction of long-term graft survival it may be required to generate allospecific Tregs *ex vivo* capable of limiting chronic allograft dysfunction. Using *in vitro* expanded Tregs with indirect pathway anti-donor allospecificity for a single MHC class I, my group demonstrated the induction of donor-specific transplantation tolerance in a murine skin transplant model following thymectomy and partial T cell depletion (Golshayan et al. 2007). Furthermore, using TCR gene transduction, Treg lines were generated *in vitro*, with both direct and indirect pathway allospecificities and it was shown that these Tregs are very effective at inducing indefinite survival of MHC-mismatched heart allografts (Tsang et al. 2009). While lines with direct or indirect specificity could prolong graft survival, indirect allospecificity was necessary to prevent chronic vasculopathy (Tsang et al. 2009). Moreover, Joffre et al. have provided additional evidence that Tregs with direct allospecificity alone cannot protect against chronic rejections and that both specificities are necessary (Joffre et al. 2008). Based on such findings, therefore, Tregs with defined indirect allospecificity are likely to be important in long-term allograft survival.

It is noteworthy to mention that although the expansion of direct-pathway allospecific human Tregs has been achieved (Peters et al. 2008), expansion of indirect pathway

Tregs has proven more difficult. This is due to the tendency of autoreactive Tregs to expand, a consequence of the technique used to generate these Tregs (i.e. autologous APCs pulsed with alloantigen) (Jiang et al. 2003, Jiang et al. 2006). Several methods have been reported that may help to circumvent this. They include either tetramer sorting (Jiang et al. 2006) or pre-use of artificial APCs (Masteller et al. 2005). An additional approach from my laboratory involves the retroviral transduction of genes encoding a known TCR with indirect specificity for alloantigen (Tsang et al. 2006).

Use of currently available humanised mouse models of allotransplantation (Shultz et al. 2007, Issa et al. 2010, Nadig et al. 2010, Wu et al. 2013, Xiao et al. 2014) has further reinforced the importance of antigen specific Tregs in this settings. These models are based on the reconstitution of immunodeficient mice with human immune cells. More recently my lab has shown the efficacy of human Tregs with direct allospecificity in preventing alloimmune dermal tissue injury in a humanised mouse model of skin transplantation (Sagoo et al. 2011). In these studies, the majority using Tregs with direct allospecificity, it was concluded that donor antigen specific Tregs are more effective as compared to polyclonal Tregs.

In addition to the evidence supporting the importance of antigen-specific Tregs in preventing solid organ rejection, after BMT donor-specific Tregs have been shown to preserve graft-versus tumor activity, whilst inhibiting graft versus host disease (GvHD) (Edinger et al. 2003). However, further studies in the context GvHD have reported that the transfer of Tregs enriched for alloantigen-specificity showed only moderately improved efficacy when compared to polyclonal Treg cell populations

(Trenado et al. 2003). This may reflect the fact that GvHD is a systemic disease and the concentration of Tregs at a localised site is not required. Based on these studies and the lack of antigen-specific requirement for the transferred Treg cells within this particular transplantation setting, phase I clinical trials, using polyclonal Tregs following haematopoietic stem cell transplantation have been initiated (reviewed in section 1.4.1).

Such adoptive transfer experiments in rodents, therefore, support the notion that tolerance requires 'tipping the balance' between reactivity and regulation. Despite such data generated in preclinical animal models successfully showing that Tregs can induce and maintain transplantation tolerance, we currently face many challenges in the laboratory that have hindered the widespread application of Treg cell therapy in the transplant setting. In addition, a number of different strategies have been proposed for the isolation and expansion of Tregs for cellular therapy. However, there is no consensus on the optimal process and many such processes have their limitations (discussed in section 1.3).

1.3 Clinical Application of autologous ex vivo expanded regulatory T cells

1.3.1 Challenges with large-scale manufacture

1.3.1.1. Regulatory T cell Isolation

The absence of a defining Treg-specific cell surface marker has led to significant research centered on the identification of a population of Tregs most suitable for clinical translation.

Two different combinations of markers have been proposed to be promising for the isolation of a pure Treg population. The first seeks to isolate $CD4^{+}CD25^{Hi}$ Tregs with the addition of an antibody to select for $CD45RA^{+}$ cells and so eliminate antigen experienced or memory T cells (Hoffmann et al. 2006) (discussed previously in section 1.2.3). Moreover, this so-called naïve Treg population yields Tregs with a greater suppressive capacity than total $CD25^{Hi}$ cells (Hoffmann et al. 2009) and have the greatest expansion potential (Hoffmann et al. 2006). Furthermore, after three weeks of *in vitro* expansion the $CD45RA^{+}$ expanded Tregs remained demethylated at the TSDR region, confirming their stability during expansion (Hoffmann et al. 2009, Putnam et al. 2009).

Despite such studies one drawback is that the number of naïve Tregs decline in the peripheral blood with age (Seddiki et al. 2006) and hence isolation based on this approach may prove to be impractical. The second approach still uses the fundamental $CD4^{+}CD25^{hi}$ phenotype to isolate Tregs but also includes CD127 expression (section

1.2.3). The rationale for using CD127 as a marker for Treg isolation is on the basis that in human Tregs there is a reciprocal expression of CD127 and FOXP3 and thus CD127 provides a sortable surrogate marker for $FOXP3^+$ Tregs (Liu et al. 2006). Moreover, two elegant studies (Booth et al. 2010, Issa et al. 2010, Nadig et al. 2010) support the *in vivo* superiority of the $CD4^+CD25^+CD127^{Lo}$ Tregs in regulating alloreactivity compared to Tregs isolated based on expression of CD4 and CD25 alone. Such studies have important implications for the design of future clinical studies.

The search for further surface markers to aid the isolation of purer or more potent Treg populations, led to studies investigating markers such as CD121a/CD121b, TGF-β/LAP (Tran et al. 2009). However, all these proteins are only expressed on activated Tregs and would only be of use to re-isolate Tregs after expansion. This may not be feasible in view of the costs of re-isolating billions of Tregs on a per-patient basis. Other studies complicate the story even further. Ito et al. showed that $FOXP3^+$ Tregs could be grouped into two subsets based on the expression of the inducible T cell co-stimulator (ICOS) (Ito et al. 2008). They showed that whilst $ICOS^-FOXP3^+$ Tregs mediate their suppressive function via TGF-β, $ICOS^+FOXP3^+$ Tregs additionally secrete IL-10.

Such studies, therefore, paint a complicated picture that when choosing the Treg marker for cell isolation we should also bear in mind other factors other than just purity, i.e. isolating potent cells with mechanism of action to suppress the immune response of interest and cells with the desirable expansion profiles.

Despite this, however, what limits choice when devising a clinically applicable protocol is that isolation techniques need to be good manufacturing practice (GMP) compliant and GMP purification reagents for all the various markers outlined above are not yet available.

The clinical Treg selection protocols to date used in the UK have used a combination of depletion and positive selection steps with the isolation tools mainly involving the automated CliniMACS plus system (Miltenyi Biotec, Bisley, United Kingdom). This enables GMP-compliant cell selection by magnetic bead-activated cell sorting (Wichlan et al. 2006). More specifically for the production of $CD4^{+}CD25^{Hi}$ Tregs, the GMP grade antibodies available enable the depletion of CD19/CD8 expressing immune cells followed by a positive selection of CD25 expressing T cells (Di Ianni et al. 2009). The major drawback with such techniques is that this process does not guarantee the selection of $CD25^{Hi}$ cells compared to the FACS sorter, which allows the important distinction to be made between the $CD4^{+}CD25^{Hi}$ and $CD25^{Int}$ cells. In addition, the process does not allow the selection of Tregs based on multiple parameters and the ~ 60% purity of the isolated cells (Peters et al. 2008) is not comparable with the >95% purity achieved using the FACS sorter (Putnam et al. 2009). Despite the drawbacks of the current process available in the UK efforts have been made to establish new GMP-grade antibodies to improve the production of Tregs for clinical trials i.e. the recently developed GMP grade CD45RA microbeads (Miltenyi Biotech) for the positive selection of $CD45RA^{+}$ immune cells (from the $CD4^{+}CD25^{+}$ population) to further refine the current markers of isolation to $CD4^{+}CD25^{+}CD45RA^{+}$.

1.3.1.2. REGULATORY T CELL EXPANSION

1.3.1.2.1. POLYCLONAL REGULATORY T CELL *EXPANSION;*

One of the obstacles in the implementation of clinical protocols for adoptive Treg cell therapy is their relative low frequency in the circulation, with Tregs forming only around 1-3% of total peripheral blood $CD4^+$ T cells. This means that for cellular therapy, it will almost certainly be necessary to expand these cells *ex vivo*, to clinically relevant numbers, prior to their administration. It has already been demonstrated that Tregs can be readily expanded using antiCD3/CD28 coated beads, supplemented with IL-2 (Levings et al. 2001, Hoffman et al 2004 and Putnam et al 2009). However, under theses circumstances, effector cells have the potential to proliferate vigorously, posing a major problem for MACS-purified $CD4^+CD25^+$ Tregs, as they are often contaminated with $CD25^+FOXP3^-$ cells. As such, studies highlight the importance of supplementing cultures with rapamycin.

This drug inhibits the mammalian target of rapamycin (mTOR), which is downstream of phosphatidylinositol 3-kinase (PI3K, a signalling molecule activated by CD28 or IL-2 receptor engagement in T cells (Thomson et al. 2009). IL-2 receptor engagement activates both PI3K-mTOR and Janus kinase-STAT pathways. Biochemical analysis of IL-2 signalling in Tregs has shown that the PI3K-mTOR pathway is attenuated, whereas the Janus Kinase-STAT pathway remains intact, suggesting that Tregs preferentially signal through the latter thus conferring their resistance to mTOR inhibition (Zeiser et al. 2008). In agreement, genetic ablation and cellular experiments that demonstrate mTOR deficiency or the addition of rapamycin, favors the outgrowth and function of Tregs (Battaglia et al. 2005, Delgoffe et al. 2009). Consistent with these *in vitro* observations, it has been shown that rapamycin can potentiate the ability

of Tregs to inhibit transplant arteriosclerosis in a humanised mouse system (Hester et al. 2012). Furthermore, in transplant patients the use of rapamycin-based immunosuppression is also associated with an increase in Tregs as compared with patients on calcineurin inhibitors (CNI) (Segundo et al. 2006, Noris et al. 2007). Thus, by favouring Treg survival and expansion and preventing the outgrowth of contaminating effector T cells (Basu et al. 2008, Zeiser et al. 2008), rapamycin ensures the growth of a pure Treg population.

Despite these advances, large scale manufacture of Tregs remains challenging in view of studies reporting that even highly pure Tregs lose FOXP3 expression with repeated stimulation, even in the presence of rapamycin (Hoffmann et al. 2009, Hippen et al. 2011). It is believed that the loss of FOXP3 is likely owing to destabilization of FOXP3 expression in Tregs (Hoffmann et al. 2009). However, the cellular and molecular basis of Treg destabilization during *in vitro* stimulation is presently unclear.

1.3.1.2.2. ALLOANTIGEN SPECIFIC REGULATORY T CELL *EXPANSION*

Studies have shown that the frequency of direct alloreactive Tregs to be between 1% and 20% (Lin et al. 2008). Proof of principle experiments have shown that Tregs with direct allospecificity can be expanded using donor APCs such as DCs and unfractionated peripheral blood mononuclear cells (Peters et al. 2008, Chen et al. 2009, Sagoo et al. 2011, Tran et al. 2012).

Moreover, recent reports have highlighted the effectiveness of CD40 activated B cells in the induction and expansion of antigen specific Tregs *in vitro* (Adachi and Ishii 2002, Tu et al. 2008, Zheng et al. 2010). As such, one of the aims of this thesis was to

develop a GMP compatible protocol, using CD40L activated allogeneic B cells to selectively stimulate the expansion of alloantigen-reactive Tregs (See Chapter 5).

Although, most successes in expanding human alloantigen specific Tregs have been in generating Tregs with direct allospecificity, efforts have also been directed to the expansion of human Tregs with indirect allospecificity (Veerapathran et al. 2011).

1.4. Clinical Application of regulatory T cells; Past experience and the Future

1.4.1. Regulatory T cell Immunotherapy in Bone marrow transplantation and type I diabetes

In spite of the potential concerns and controversies outlined with regards to Treg isolation and expansion protocols, as of April 2013 four clinical trials of Treg therapy in humans have been reported, three in the setting of GvHD and one in Type I diabetes, with data summarised below.

Beneficial effects of Treg infusions on allograft survival were first described in bone marrow transplantation models in which donor Tregs reduced the incidence of GvHD. The first human trial using Treg cell therapy conducted by Trzonkowski et al. involved two patients. The first patient had chronic GvHD two years post bone marrow transplantation. After receiving $0.1x10^6$/kg FACS purified *ex vivo* expanded Tregs from the donor, the symptoms subsided and the patient was successfully withdrawn from immunosuppression without evidence of recurrence. The second patient had acute GvHD at one-month post transplantation, which was treated with several infusions of expanded donor Tregs. Despite the initial and transitory

improvement, the disease progressed and ultimately resulted in the patient's death (Trzonkowski et al. 2009). This was the first report to show that adoptive transfer of Tregs is well tolerated and thus was a major breakthrough.

Results of a larger phase I/II study were reported in which a total of 23 patients receiving umbilical cord blood (UCB) stem cell transplants were enrolled into a Treg escalation trial (Brunstein et al. 2011)**.**

$CD4^{+}CD25^{Hi}$ Tregs were isolated from a third party UCB graft and expanded polyclonally with antiCD3/CD28 coated beads and recombinant IL-2 over a period of 18 days. Patients received expanded Tregs at doses ranging from 1 x 10^5/kg to 30x10^5/kg. Of note, the targeted Treg dose was only achieved in 74% of cases. Compared with the 108 historical controls, there was a reduced incidence of grades II-IV acute GvHD (from 61% to 43%; p=0.05), although the overall incidence of GvHD was not significantly different.

In a third trial (Phase I/II) conducted by Di Ianni et al. 28 patients who had received haematopoietic stem cell transplantation for haematological malignancies were enrolled (Di Ianni et al. 2011). Patients received donor Tregs without *ex vivo* expansion and donor effector T cells (Teff) without any other adjuvant immunosuppression. Different dose regimens were used, ranging from 5x10^5/kg Teffs with 2x 10^6/kg Tregs to 2x10^6/kg Teffs with 4x10^6/kg Tregs. As two patients receiving the latter regimen developed acute GvHD, compared with none of the other patients, the authors concluded that a dose of 1x10^6/kg Teffs with 2x10^6/kg Tregs to be safe. Moreover, patients receiving Tregs demonstrated accelerated immune reconstitution, reduced CMV reactivation, and a lower incidence of tumour relapse and GvHD when compared to historical controls. However, it is also important to note

the disappointing patient survival with only 13 out of the 26 patients surviving, however, this may have been due to prior exposure to fungal infections and the harsh conditioning regimens.

In the trial of Type I diabetes, Marek-Trzonkowska et al. presented the data on the one year follow up of 12 children with the condition, treated with autologous expanded *ex vivo* Tregs. Patients received either a single or double Treg infusion up to a total dose of $30x10^6$/kg. The data supported the safety of the infused Tregs, with 8/12 treated patients requiring lower requirements of insulin, with two children completely insulin independent at one year (Marek-Trzonkowska et al. 2014).

1.4.2. Regulatory T cell Immunotherapy in Solid Organ Transplantation

The results of the trials to date have highlighted the favourable safety profile of freshly isolated and polyclonally expanded Tregs with varied reports of efficacy. As a result, the prospects of Treg adoptive cell therapy are now widely recognised with the information gleaned from these preliminary trials now guiding the clinical progression of these cells into the realms of organ transplantation.

This has seen the start of two trials at Guy's Hospital;

1. To prevent renal transplant rejection as part of the ONE Study (NCT02129881), a multicentre Phase I/II study funded by the European Union FP7 programme. This trial will investigate the safety of and potential efficacy of infusing *ex vivo* expanded Tregs, amongst other regulatory cells.

2. To prevent liver transplant rejection, ThRIL (NCT02166177), described in detail in section 1.4.2.1.

1.4.2.1. THRIL; REGULATORY T CELL IMMUNOTHERAPY IN LIVER TRANSPLANTATION

Liver transplantation remains the treatment of choice for patients with end stage liver disease. Despite improvements in short term outcome, the obligatory protracted use of powerful non-specific immunosuppressants has led to an accelerated rise in morbidity and mortality as a result of chronic rejection and associated toxicity. As such, the current standing on immunosuppression in transplantation is far from ideal. As a result there has been enormous interest in the minimization/complete withdrawal of immunosuppressive drugs in liver transplant recipients.

As eluded to in earlier section 1.1.1 there have been reports in the literature that a variable proportion of liver transplant recipients develop a state of 'operational' tolerance thus forgoing the requirements of therapeutic immunosuppression. This phenomenon, however, only occurs late after transplantation. It is, therefore, necessary to find novel strategies to accelerate the spontaneous development of tolerance early after transplantation and in turn negate the use of lifelong immunosuppression.

The liver itself, is an inherently tolerogenic organ, requiring a less aggressive immunosuppressive regimen post transplantation as compared to other organ allografts. Furthermore, acute cellular rejection in liver transplantation, albeit common, is readily treated and resolves without long-term sequelae (Lerut and

Sanchez-Fueyo 2006). In view of these features the liver proves to be an ideal candidate in which trials of Treg immunotherapy can be initiated.

In this regard, the first combined Phase I/IIa clinical trial of Treg immunotherapy worldwide in the setting of liver transplantation, ThRIL (NCT02166177) has been initiated at King's College London. Here, the safety, tolerability and efficacy of polyclonally expanded Tregs in combination with depletion of alloreactive T cells and short-term immunosuppression will be assessed.

One of the key prerequisites for the success of a trial such as ThRIL is data on the characteristics of Tregs isolated from the patients who will be receiving the therapy post transplantation. In addition, for the feasibility of such a trial it is imperative to demonstrate that these cells can be expanded to reach numbers suitable for their clinical application, with maintenance of their phenotype and function post expansion.

In this regard, in this thesis data is presented on the isolation, characterisation and expansion of Tregs from patients with alcohol related cirrhosis (ARC) since this diagnosis accounts for 80% of all liver cirrhosis cases in the UK and the majority of patients on the liver transplant waiting list. As such section 1.5 reviews the literature on the pathogenesis of this condition, an understanding of which is of importance in view of devising novel therapies in this setting.

1. 5. End Stage Liver disease; Alcohol Related Cirrhosis

1.5.1. Classification of progression

Chronic liver diseases derive from a wide array of pathological origins including: hepatitis B/C (HBV/HCV), alcoholic liver disease (ALD) and non-alcoholic fatty liver disease (NAFLD). Of these ALD is the major cause of chronic liver disease in the western world (Walsh and Alexander 2000). The disease spectrum of ALD can be classified according to an evolution of histological stages progressing initially from steatosis, to hepatitis and culminating in cirrhosis, however, the sequence of these events varies considerably amongst different individuals. Whilst almost all heavy drinkers develop steatosis, only around 10-35% show various degrees of alcoholic hepatitis and 8-20% progress to cirrhosis (Yip and Burt 2006) . Thus, understanding the pathogenesis of ALD and predisposition to cirrhosis not only has an important clinical, but also a social implication, since 80% of all cases of liver cirrhosis, necessitating transplantation, seen in the UK are secondary to ALD.

1.5.2. Aetiology and Pathogenesis

Although the association of excessive alcohol consumption and ALD is already clearly established the precise pathogenesis of this condition is still not fully understood. An individual's susceptibility to develop ALD is thought to be a multi-step process dependent on several factors including: genetic predisposition, environmental influences and immune mediated chronic alcohol toxicity (Seth et al. 2011). While research into the genetic predisposition and external environmental

influences have been inconclusive (Stickel and Osterreicher 2006), immunological based investigation in ALD has yielded significant data supplementing the notion that immune-mediated injury plays a predominant role in the pathogenesis of this condition.

1.5.2.1. ROLE OF IMMUNITY IN THE PROGRESSION OF LIVER DAMAGE

Whilst the role of the innate immune system in this setting has been broadly acknowledged, the influence of the adaptive immune system has received less attention (Hines and Wheeler 2004). However, early studies have shown that the neutrophil-rich liver infiltrates, characteristic of alcoholic hepatitis, contain both $CD4^+$ and $CD8^+$ T lymphocytes (Chedid et al. 1993). Furthermore, studies from alcohol-treated mice and chronic drinkers have concluded that liver infiltrating T cells express an activation/memory phenotype which following stimulation produce pro-inflammatory Th-1 cytokines such as IFNγ and TNF-α (Song et al. 2001, Song et al. 2002). Further evidence supporting the role of adaptive immunity in ALD, stems from studies depicting the presence of circulating antibodies against alcohol altered autologous hepatocytes in ALD patients (Paronetto 1993) with further studies indicating that the antibody profile in ALD patients bears several similarities to that of autoimmune hepatitis (Perperas et al. 1981, Ma et al. 1997).

Recent studies have also detailed the role of Th17 effector lymphocytes in ALD and their implication in various autoimmune diseases. There have been reports of the presence of these cells in both the liver and circulation of patients with ALD, with the

associated IL-17 production promoting liver neutrophil infiltration during alcoholic hepatitis (Lemmers et al. 2009).

Following the results of such studies, it is pertinent to also focus research on the adaptive immune system in ALD with the work presented in this thesis, directed at understanding the role of $CD4^+CD25^+FOXP3^+$ regulatory T cells (Tregs) in this setting.

1.5.2.2. Oxidative Stress and importance of Heme Oxygenase-1

Of importance is that alcohol related liver damage is defined by chronic inflammation, hepatocellular injury and cell death. This is in part due to ethanol metabolism and oxidative stress. Studies with hepatocytes isolated from control rats and from alcohol fed rats, indicated that the enzymatic reaction of alcohol metabolism, involving aldehyde dehydrogenase, results in the increased production of reactive oxygen species (ROS), hepatocyte injury and apoptosis. Moreover, all of these reactions could be blocked by administration of antioxidants (Adachi and Ishii 2002, Bailey and Cunningham 2002).

Based on such data and in view of the importance of oxidative stress in the development of liver cirrhosis (Poli 2000, Natarajan et al. 2006) it is of significance to determine whether alterations in the pathway important in maintaining antioxidant and oxidant homeostasis is altered in ARC and the relevance of this in the perpetuation of the disease.

Heme oxygenase (HO) is the rate limiting enzyme involved in the metabolism of heme into biliverdin, carbon monoxide (CO) and free iron (Fe^{2+}) (Choi and Alam 1996) **(Figure 1.6).** Three isoforms of HO have been described; HO-1 is peripherally induced whereas HO-2 and HO-3 are constitutive isozymes. In general, the expression of HO-1 is relatively low with its upregulation evident during times of cellular stress, constituting a critical cytoprotective mechanism with anti-apoptotic, anti-proliferative properties with an important role in regulating T cell responses. In this regard, HO-1 knock-out mice exhibit a state of chronic inflammation characterised by splenomegaly, lymphadenopathy and lymphocytosis, confirming the immunoregulatory roles of HO-1 (Poss and Tonegawa 1997, Poss and Tonegawa 1997). In parallel, studies administrating an HO-1 inducer to mice report the suppression of T cell mediated cytotoxicity and Th1 mediated cytokine production (Woo et al. 1998).

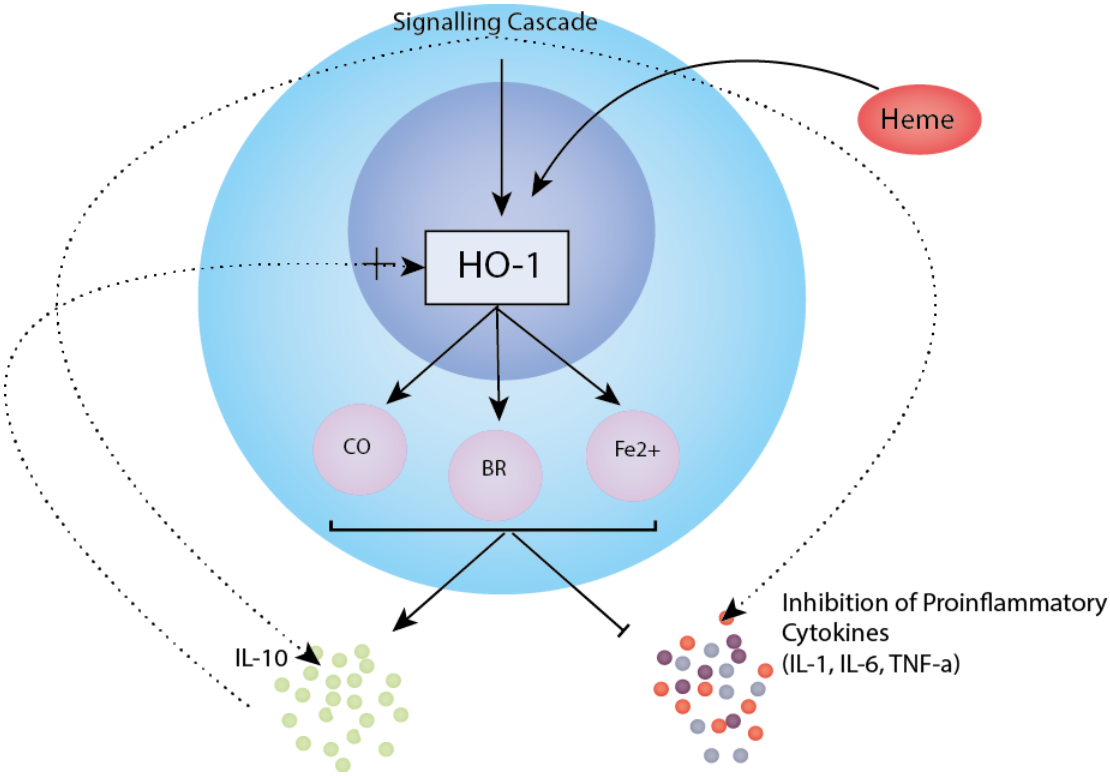


FIGURE 1.5. SCHEMATIC DIAGRAM REPRESENTING THE ENZYMATIC ACTIVITY OF HO-1 AND ITS ANTI-INFLAMMATORY PRODUCTS.

Oxidative stress results in the generation of free heme. HO-1 metabolises the heme into equimolar products of Fe^{2+}, CO and biliverdin. The biliverdin is subsequently converted to bilirubin by biliverdin reductase and the free Fe^{2+}, bound and sequestered as ferritin. Both CO and bilirubin have major anti-apoptotic/ antioxidant properties playing a critical role in the balance of inflammatory reactions. *Abbreviations: BR- bilirubin; CO- carbon monoxide; Fe^{2+}- ferrous iron; IL- interleukin; TNF-α- tumor necrosis factor-α.* Adapted from (Paine et al. 2010)

The role of HO-1 in modulating various human immune-dominated chronic inflammatory diseases has also been widely reported (Maines 1997, Song et al. 2002, Almolki et al. 2004, Ryter and Choi 2005, Motterlini et al. 2012). It is now broadly accepted that HO-1 can be induced in a variety of different cell types, although the expression of this enzyme in T cells is not fully understood, with reports that HO-1 is differentially expressed by $CD4^{+}CD25^{-}$ effector T cells (Teff) and Tregs (Hori et al. 2003, Pae et al. 2003). Moreover, data from murine models provide contradictory

evidence for the role of HO-1 in Treg suppressor function warranting further research. (Zelenay et al. 2007, Schumacher et al. 2012, Xiao et al. 2014). As such in the present study I sought to characterise the Tregs from patients with ARC and to determine whether the HO-1 pathway can account for the Treg functional properties in this cohort of patients.

1.6 General hypothesis and objectives

The current standing on immunosuppression in transplantation is far from ideal. As a result there has been enormous interest in the minimization/complete withdrawal of immunosuppressive drugs in liver transplant recipients. Bearing in mind the integral role of Tregs in promoting immune homeostasis this thesis hypothesizes that Tregs are ideal candidates for tolerance induction in the setting of liver transplantation.

This hypothesis is investigated by pursuing the following aims:

1) To conduct an in-depth phenotypic and functional characterisation of Tregs isolated from prospective liver transplant recipients. .
2) To utilize a GMP compatible isolation and expansion protocol for the polyclonal expansion of clinical grade patient-derived Tregs for cell therapy application.

Evidence already exists in support of graft specific Tregs as compared to polyclonal Tregs in the setting of transplantation. As such further objectives of this thesis included:

3)

   a) To devise a protocol for the clinical-grade manufacture of human alloantigen specific Tregs.
   b) To compare the *in vivo* function of antigen specific and polyclonally expanded Tregs, using a humanized mouse model of skin transplantation.

Chapter 2

MATERIALS AND METHODS

2.1. List of Reagents and Suppliers

Chemicals and Solutions		
Reagents	Supplier	
3,3',5,5' – tetramethylbenzidine (TMB substrate solution)	Zymed	Paisley, UK
Bovine Serum Albumin (BSA)	Sigma Aldrich	St Luis, MO, USA
CellTrace™ CFSE	Invitrogen	Paisley, UK
CD8 Microbeads (Research grade)	Miltenyi Biotec	Woking, UK
CD25 Microbeads (Research grade)	Miltenyi Biotec	Woking, UK
Dimethyl sulfoxide (DMSO)	Sigma Aldrich	St Luis, MO, USA
Dynabeads® anti-CD3/CD28 T cell expander	Invitrogen	Paisley, UK
Ethylenediaminetetraacetic acid (EDTA)	Invitrogen	Paisley, UK
Fixation/permeabilisation solution	eBioscience	San Diego, CA, USA
Foetal Calf Serum (FCS)	Gibco	Paisley, UK
Human AB serum (HS)	Invitrogen	Paisley, UK
Ionomycin	Sigma Aldrich	St Luis, MO, USA
Lymphoprep™	Nycomed Pharma	Zürich, Switzerland
Monensin	eBioscience	San Diego, CA, USA
Phorbol myristate acetate (PMA)	Sigma Aldrich	St Luis, MO, USA
1X Sterile Phosphate Buffered Saline (PBS)	Life Technologies	Paisley, UK

Rapamycin	Pfizer	New York City, USA
Radio-Immunoprecipitation Assay (RIPA) Cell Lysis Buffer	Sigma Aldrich	St Luis, MO, USA
Recombinant human IL1-β	R&D systems	Abingdon, UK
Recombinant human IL-2	R&D systems	Abingdon, UK
Recombinant human IL-6	R&D systems	Abingdon, UK
Recombinant human IL-21	Cell Sciences	Canton, MA, USA
Recombinant human IL-23	R&D systems	Abingdon, UK
Recombinant human TGF-β	R&D systems	Abingdon, UK
RosetteSep® Human $CD4^+$ T Cell Enrichment Cocktail	Stemcell Technologies	Manchester, UK
RPMI-1640	Gibco	Paisley, UK
Sulphuric acid (H_2SO_4)	R&D systems	Abingdon, UK
Trypan-blue	Sigma Aldrich	St Luis, MO, USA
Tween® 20	Sigma Aldrich	St Luis, MO, USA
X-vivo 15	Lonza	Basel, Switzerland

Antibodies					
Specificity	Conjugate	Clone	µL/test	Supplier	
CCR6	PeCy7	R6H1	3	eBioscience	San Diego, CA, USA
CD4	PercP	SK3	5	BD Bioscience	Oxford, UK
CD8	PeCy7	SK1	5	eBioscience	San Diego, CA, USA

CD25	APC	2A3	5	BD Bioscience	Oxford, UK
CD25	PE	CD25-4E3	3	eBioscience	San Diego, CA, USA
CD27	eFluor450®	O323	2	eBioscience	San Diego, CA, USA
CD39	PeCy7	eBioA1	2	eBioscience	San Diego, CA, USA
CD45 RA	FITC	JS-830	2	eBioscience	San Diego, CA, USA
CD62L	PeCy7 FITC	DREG-56	2	eBioscience	San Diego, CA, USA
CD127	eFluor450®	eBioRDR5	2	eBioscience	San Diego, CA, USA
CD161	eFluor450®	HP-3G10	3	eBioscience	San Diego, CA, USA
CD178 (FAS-L)	PE	NOK-1	2	Biolegend	San Diego, USA
CD274 (PDL-1)	PeCy7	MIH-1	3	eBioscience	San Diego, USA
CTLA-4 (CD152)	PE	14D3	3	eBioscience	San Diego, USA
CXCR3	Pacific Blue	G025H7	3	Biolegend	San Diego, USA
FOXP3	FITC	236A/E7	5	eBioscience	San Diego, USA
FOXP3	PE	236A/E7	5	eBioscience	San Diego, USA
GARP	eFluor450®	G14D9	5	eBioscience	San Diego, USA
GITR	PE	eBioAITR	5	eBioscience	San Diego, USA
Granzyme-B	PE	GB11	5	eBioscience	San Diego, USA

Helios	PE	22F6	5	Biolegend	San Diego, USA
HLA-DR	PeCy7	LN3	2	eBioscience	San Diego, USA
ICOS-L (B7RP1)	PE	MIH12	3	eBioscience	San Diego, USA
ICOS (CD278)	PE	ISA-3	3	eBioscience	San Diego, USA
IFN-γ	PeCy7	4S.B3	1.5	eBioscience	San Diego, USA
IL17	PE	eBio64DEC17	2	eBioscience	San Diego, USA
PD-1	PE	eBioJ105	3	eBioscience	San Diego, USA
TIM-3	PE	344823	2	R&D systems	Abingdon, UK
Galectin-9 (Ecalectin)	Unlabeled	ECA42	1.2	MBL	Nagoya, Japan
IgG2b	PE	M32404	5 (1:10)	Life Technologies	Paisley, UK

Kits		
Minimacs CD4+CD25+ T regulatory cell Isolation Kit	Miltenyi Biotec	Woking, UK
ImmunoSet™ HO-1 (human), ELISA development kit	Enzo Life Sciences	Exeter, UK
IL-17 Duo-Set ELISA kit	R&D systems	Abingdon, UK

Consumables and Instruments		
96-well round-bottom plate	BD Falcon	Oxford, UK
24- well round-bottom plate	BD Falcon	Oxford, UK
96-well ELISA microplate	BD Falcon	Oxford, UK

Bio-Tek EL800 Automatic Plate Reader	Wolf Laboratories	Pocklington, UK
FACSAria™ II cell sorter	BD Biosciences	Oxford, UK
FACSCaliber™ II flow cytometer	BD Biosciences	Oxford, UK
LSRFortessa™ Flow Cytometer	BD Biosciences	Oxford, UK
Filtermate 96, multi-channel harvester	PacKard Bioscience	Pangbourne, UK
LD columns	Miltenyi Biotec	Woking, UK
LS columns	Miltenyi Biotec	Woking, UK
MS columns	Miltenyi Biotec	Woking, UK

Software		
FlowJo software	Tree Star Inc	OR, USA
GraphPad Prism® 5 software	GraphPad	La Jolla, CA, USA

TABLE 2.1. LIST OF REAGENTS AND SUPPLIERS.

The volume of antibody indicated in the table was chosen according to preliminary experiments using PBMCS, in which the volume recommended by the manufacturer was titrated and found to exert readily detectable and specific staining.

2.2. SUBJECTS

King's College Hospital Research Ethics Committee approved the study and written informed consent was obtained from each subject prior to enrollment into the study.

	Alcohol related Cirrhosis Patients	Non-Alcoholic Steatohepatitis	Healthy Controls
Number	20	5	20
Sex (M:F)	18:2	3:2	18:2
Average Age (Years)	55.5±8.87	61.4 ± 5.87	55.5±8.87
Average MELD score	13.3 ± 0.795	9.60 ± 2.16	N/A

TABLE 2.2. SUBJECT DEMOGRAPHICS

Data presented as the mean ± standard deviation (SD). MELD is a scoring system for assessing the severity of chronic liver disease and is now widely used for prioritizing receipt of a liver transplant. MELD uses the patient's laboratory values for bilirubin, serum creatinine and the international normalized ratio for prothrombin time (INR) to predict three month survival. In patients with cirrhosis, an increasing MELD score is associated with increasing severity of hepatic dysfunction and increased three-month mortality risk. It is calculated according to the following formula: MELD= **3.8 x [Ln(Bilirubin)] + 11.2 x [Ln(INR)] + 9.6 x [Ln(creatinine)] + 6.4.** *Abbreviations: M:Male; F:Female; N/A: not applicable; MELD: Model for End-Stage Liver Disease.*

2.2.1. CIRRHOTIC PATIENTS

A total of 25 patients with liver cirrhosis on the transplant waiting list at King's College Hospital were included in this study. Of these patients 20 were diagnosed with alcohol related cirrhosis (ARC, having been abstinent for at least 6 months) and 5 patients with Non-Alcoholic Steatohepatitis (NASH, served as disease controls). Inclusion criteria was set to include patients: able to give written informed consent, diagnosed with end-stage liver disease and listed for primary liver transplant; with a MELD score of ≤ 25 at the time of transplantation assessment.

Patients with HIV or RNA-positive Hepatitis C Virus infection, autoimmune liver disease, previous organ transplants, Epstein-Barr Virus and/or Cytomegalovirus sero-negativity, chronic use of systemic immunosuppressants, hepatocellular carcinoma outside Milano criteria and leukocytes $<1.5x10^9$/L and/or platelets $<50x10^9$/L were excluded.

	Alcohol-Related Cirrhosis Patients	Non-Alcoholic Steatohepatitis
Number of Patients	20	5
Hb (nv: 115-180g/l)	120 (83.0-144)	109 (99.0-133)
Platelets (nv: 150-400x10^9/l)	93.5 (39.0-415)	88.0 (63.0-241)
INR (nv: 0.9-1.1)	1.53 (1.04-3.31)	1.32 (1.05-1.45)
Bilirubin (nv: <20μmol/l)	40.5 (7.00-137)	57.0 (8.00-134)
ALP (nv: 40-165U/l)	120 (46.0-242)	101 (78.0-220)
AST (nv: <50U/l)	42.0 (19.0-94.0)	63.0 (43.0-77.0)
γGT (nv: <60U/l)	51.5 (17.0-365)	270 (147-439)
Albumin (nv: 35-50g/l)	33.5 (23.0-42.0)	32.5 (29.0-47.0)
Creatinine (nv: 50-120μmol/l)	76.0 (33.0-135)	68.0 (37.0-82.0)

TABLE 2.3. PATIENT LABORATORY DATA

Data are presented as the median (range). *Abbreviations: ALP- Alkaline phosphatase; AST- Aspartate transaminase; γGT- gamma glutamyl transferase; INR- International Normalised Ratio; nv- normal values.*

2.2.2. HEALTHY SUBJECTS

20 age and sex matched Healthy subjects, who were clinically well for at least 2 months, served as controls (Healthy controls, HC).

2.3. PERIPHERAL BLOOD MONONUCLEAR CELL (PBMC) ISOLATION

150 ml of whole blood, obtained from peripheral vein venipuncture, was diluted in sterile PBS at a ratio of 1:2 and carefully layered onto 20 ml of Lymphoprep™. The blood was then centrifuged at 2000 rpm for 20 minutes at 20^{o}C. The mononuclear cell layer was next collected following aspiration of the cell layer at the interface and washed with sterile PBS twice at 1200 rpm and 1800 rpm respectively for 10 minutes at 4^{o}C. Isolated PBMCs were subsequently further purified through various different methods as follows:

2.4. REGULATORY T-CELL PURIFICATION

2.4.1. GMP PURIFICATION PROTOCOL

$CD4^{+}CD25^{+}$ Tregs were immunomagnetically isolated from freshly isolated PBMCs by CD8 depletion followed by CD25 positive selection, using GMP compatible reagents. The protocol is described in detail below.

2.4.1.1. $CD4^+CD25^+$ ISOLATION

2.4.1.1.1. $CD8^+$ DEPLETION

PBMCs were first resuspended in MACS buffer (PBS containing 0.5% FCS and 2 mM EDTA), at 10 ml/10^9 total cells, and incubated with CD8 microbeads, 750 µL/10^9 total cells, for 30 minutes in the dark at room temperature, on a roller. Post incubation, the bead bound cells were washed in MACS buffer by adding 1-2ml of MACS buffer for 10^7 cells and centrifuged at 1800 rpm for 10 minutes. Subsequently the supernatant was aspirated completely. The cells were next resuspended in 500 µL of MACS buffer per 10^8 cells and subsequently passed through MACS LS columns contained within a magnet to remove the CD8 microbead labeled cells. Of note, the LS columns were prepared prior the application of the cell suspension, by rinsing with 3ml of MACS buffer.

Post application of the cell suspension onto the column, the unlabeled cells were collected below and the column washed three times with 3 ml of MACS buffer. The effluent, containing the unlabeled cells, was collected and washed with MACS buffer.

2.4.1.1.2. $CD25^+$ ENRICHMENT

The cells were subsequently resuspended in MACS buffer (20 ml/ 10^9 total cells) and incubated with CD25 microbeads (750 µL/10^9 total cells) for 15 mins at 4^oC in the dark. Cells were next washed with MACS buffer, by adding 1-2ml of buffer per 10^7 cells and centrifuged at 1800 rpm for 5 minutes. The cells were next resuspended in 500 µL of MACS buffer per 10^8 cells and passed through MACS LS columns contained within a magnet, to isolate CD25 microbead labeled cells. Of note, prior to

the application of the cell suspension, the column was placed in the magnetic field of a suitable MACS separator and rinsed with 3ml of MACS buffer.

Post application of the cell suspension onto the column, the column was washed with 3ml of MACS buffer three times. Subsequently the column was removed from the magnet and placed on a suitable collection tube. The labeled cells within the column were plunged through with 2ml of MACS buffer. The $CD25^+$ labeled cells were subsequently washed in staining buffer and purity assessed by flow cytometry (**Figure 2.1** depicts the purity of the isolated cells post $CD8^+$ cell depletion and $CD25^+$ cell enrichment).

2.4.2. MINIMACS $CD4^+CD25^+$ AND $CD4^+CD25^-$ ISOLATION

Tregs were immunomagnetically separated from freshly isolated PBMCs using the Minimacs $CD4^+CD25^+$ T regulatory cell Isolation Kit. Isolated PBMCs were resuspended in MACS buffer at 90 μL per 10^7 total cells. To this suspension $CD4^+$ T Cell Biotin-Antibody Cocktail at 10 μL per 10^7 cells, was added and subsequently incubated for 10 minutes at 4^oC. After incubation, anti-Biotin microbeads, 20 μL per 10^7 cells, were added to the suspension and incubated at 4^oC for 15 minutes. MACS LD column was placed in the magnetic field of a suitable MACS separator and the column rinsed with 2ml of buffer. Subsequently the cell suspension was passed through the column. Next, the column was washed twice with 1 ml of MACS buffer and the unlabeled cells collected below, forming the pre-enriched $CD4^+$ cell fraction. The unlabeled cells were washed in MACS buffer and centrifuged at 1800 rpm for 10 minutes. The supernatant was aspirated and the cell pellet resuspended in 90μL of

MACS buffer per 10^7 cells. To this suspension 10 μL per 10^7 cells of CD25 microbeads was added and incubated for 15 minutes at 4°C in the dark. The cells were next washed by adding 1-2ml of buffer per 10^7 cells, and centrifuged at 1800 rpm for 10 minutes. Subsequently the cells were resuspended in 500 μL of MACS buffer per 10^8 cells.

Next, an MS column was placed in the magnetic field of a suitable MACS separator and the column rinsed by adding 500ml of MACS buffer. The cell suspension was then added onto the column and the effluent collected. The column was washed a further 3 times with 500 μL of MACS buffer.

The effluent, forming the $CD4^+CD25^-$ fraction was collected, washed with MACS buffer and centrifuged at 1800 rpm for 5 minutes. Subsequently, cells were counted and phenotyped (**Figure 2.1A** depicts a representative plot of purity of $CD4^+CD25^-$]. The proliferative capacity of the isolated cells was assessed by labelling with CFSE (section 2.7). Subsequently, the cells were cryopreserved for use as autologous and allogeneic Teffector cells in Treg suppression assays. Of note, cells were cryopreserved in freezing media consisting of 90% fetal calf serum (FCS) and 10% dimethyl sulphoxide (DMSO). Cryopreserved cells were frozen at -80°C (-1°C/min) and stored in liquid nitrogen.

To obtain the $CD4^+CD25^+$ cells, the MS column was removed from the magnet and flushed with 1 mL of MACS into a separate collection tube, the isolated cells were washed in staining buffer and purity of the isolated $CD4^+CD25^+$ fraction assessed by flow cytometry (**Figure 2.1B** depicts the purity of the isolated $CD4^+CD25^+$ cells using this protocol).

2.4.3. CELL SORTING OF $CD4^+CD25^+CD127^{LO}$ CELLS

RosetteSep® Human $CD4^+$ T Cell Enrichment Cocktail was added at 50 µL/mL of whole blood and incubated for 20 minutes at room temperature. Subsequently, the blood was diluted with sterile PBS at a ratio of 1:2 and 25 ml of the sample layered on 15 ml of lymphoprep and centrifuged for 20 minutes at 2000 rpm. The enriched cells were then removed from the plasma interface and washed with PBS twice. Cells were next stained with anti-CD4, anti-CD25 and anti-CD127 fluorochrome conjugated monoclonal antibodies. Tregs were subsequently sorted on the BD FACSAria™ II cytometer based on a population of $CD4^+CD25^+CD127^{Lo}$ cells (**Figure 2.1C** depicts a representative plot of the purity of sorted $CD4^+CD25^+CD127^{Lo}$ cells).

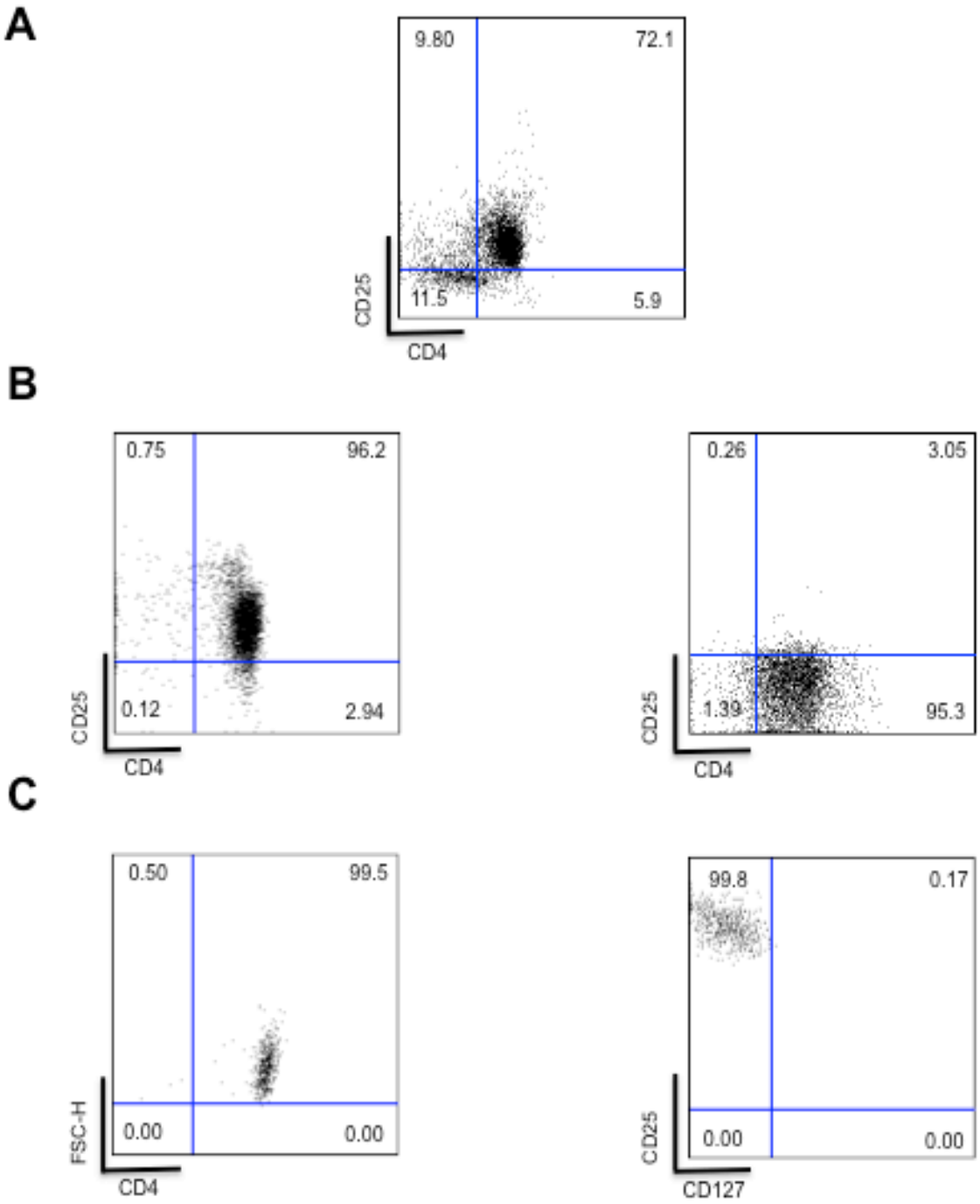


FIGURE 2.1. REPRESENTATIVE PURITIES OF REGULATORY T CELLS ISOLATED USING THE DESCRIBED TECHNIQUES.

A Shows the purity of $CD4^+CD25^+$ Tregs following isolation in concordance with a GMP compatible isolation technique involving $CD8^+$ cell depletion and subsequent $CD25^+$ cell enrichment. **B** Panel on the left shows the purity of $CD4^+CD25^+$ Tregs isolated using the Minimacs $CD4^+CD25^+$ T regulatory cell Isolation Kit, with the panel on the right showing the purity of the $CD4^+CD25^-$ fraction. **C** Displays the relative purity of $CD4^+CD25^+CD127^{Lo}$ cells following fluorescence activated cell-sorting (FACS).

2.5. FLOW CYTOMETRY

Flow cytometry is a laser-dependent biotechnology, which is utilized for the assessment and sorting of cells. Fluorescently labeled cells are suspended in a stream of fluid, passing through a laser in single file. The laser excites the cells and results in the emission of light at variable wavelengths allowing for the detection and selection of different cell types. Cells are fluorescently labeled with various fluorochrome conjugated antibodies which may be used to detect different surface or intracellular markers.

2.5.1. SURFACE STAINING

Cells were stained with the fluorochrome conjugated monoclonal antibodies to CCR6, CD4, CD8, CD25, CD27, CD39, CD45 RA, CD62L, CD127, CD161, CD178 (FAS-L), CD274 (PDL-1), TIM-3, CXCR3, GARP, GITR, HLA-DR, ICOS, ICOS-L (B7RP1), and PD-1. Cells were washed in staining buffer (PBS containing 1% FCS) and subsequently stained with monoclonal antibodies in a 96 well 'u' bottom plate (volumes stated in section 2.1). Cells were incubated for 20 minutes at 4^{o}C in the dark. Following incubation the cells were washed and stained for intracellular markers.

2.5.2. INTRACELLULAR STAINING

After extracellular staining, cells were washed and resuspended in 100 µL of Fixation/Permeabilisation solution, incubated at 4°C in the dark for 1 hour. After

washing, cells were then subject to intracellular staining to CTLA-4, FOXP3, Granzyme-B, Galectin-9, Helios with monoclonal antibodies diluted in 90 µl staining buffer and 10 µl of 1 x permeabilisation buffer (Volumes stated in section 2.1).

When staining for Galectin-9, cells were washed before the addition of a PE-conjugated anti-mouse IgG2b secondary antibody for 20 minutes. For intracellular cytokine staining of IL-17 and IFN-γ, cells were initially activated with a leukocyte activation cocktail consisting of phorbol myristate acetate (PMA) 5 ng/ml and Ionomycin 1 µg/ml, which together enhance global cytokine production, as well as Monensin 2 µM (to prevent cytokine transport from the endoplasmic reticulum to the golgi apparatus, hence favoring intracellular accumulation) and incubated for 4 - 5 hours at 37^{o}C. Following incubation, cells were resuspended in 100 µL of Fixation/Permeabilisation solution and incubated at 4^{o}C in the dark for 1hour. Cells were then washed and stained with antibodies specific to IL-17 and IFN-γ (section 2.1). Antibodies were added after dilution in 90µl staining buffer and 10 µL 1x permeabilisation buffer. After incubation for 30minutes at 4^{o}C in the dark, the cells were washed, resuspended and flow cytometric analysis conducted.

The stained cells were analysed on the BD LSRFortessa™ cytometer and the BD FACSCalibur™ cytometer with the acquire data analysed using FlowJo Software.

2.6. Regulatory T cell Cell Culture

Tregs were isolated as previously described (section 2.4.1.) using GMP compatible reagents. $CD4^{+}CD25^{+}$ Tregs were then plated at 1 x 10^{6} cells/ml, on 24-well flat bottom plates in culture media, X-vivo 15, supplemented with 5% human AB serum

(HS), and expanded in both the presence and absence of 100 nM rapamycin. Cells were polyclonally activated with anti-CD3 and anti-CD28 coated beads (Dynabeads®) at a 2:1 bead:cell ratio and cultures supplemented with IL-2 (500 IU/mL) 4 days post-activation and replenished every 2 days thereafter. Cells were restimulated every 10-12 days by magnetically removing the activation beads and adding fresh beads, rapamycin and IL-2. The expansion period was limited to 36 days with concurrent assessment of Treg phenotype and function throughout culture.

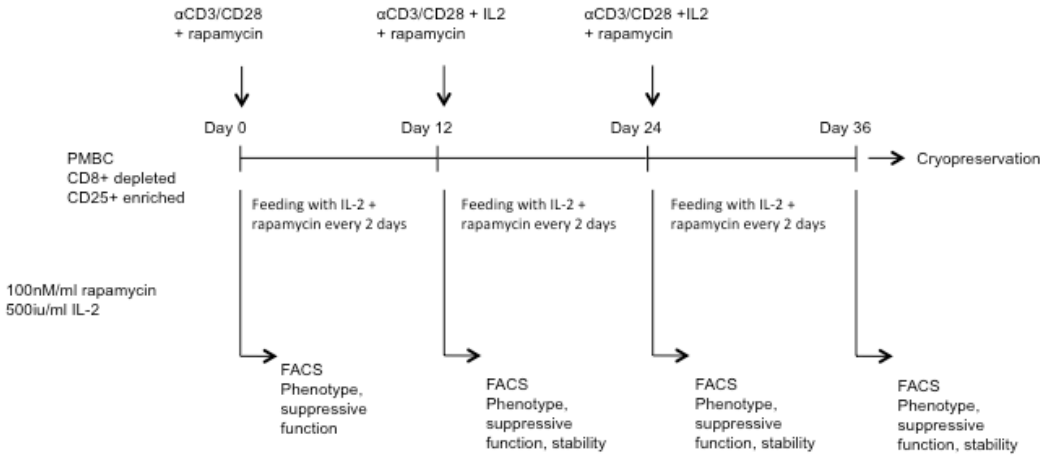


FIGURE 2.2. GMP COMPATIBLE TREG ISOLATION AND EXPANSION PROTOCOL

Abbreviations: FACS- Fluorescence-Activated Cell Sorting; IL-Interleukin; PBMC-Peripheral Blood Mononuclear Cells; S- Stimulation.

2.7. SUPPRESSION ASSAY

Before use, cryopreserved responder $CD4^+CD25^-$ T cells (Teff) (isolated as described in section 2.4.2) were thawed quickly by gently swirling cryotubes in a 37°C water bath. The cells were next washed twice in sterile PBS at 1800 rpm for 5 minutes and subsequently labeled with 2.5 nM Carboxyfluorescein succinimidyl ester (CFSE). $1x10^5$ Teffs/well were plated on 96 'u' bottom plates either alone or in co-culture with $CD4^+CD25^+$ Tregs at differing ratios (Treg:Teff - 1:1, 1:5 and 1:10) in X-Vivo 15 medium supplemented with 5% HS and activated with anti-CD3 and CD28 coated beads (Dynabeads®), at a ratio of 1:42 (bead:cell). In each case the number of Teffs was kept constant and the number of Tregs titrated down with the total volume of each well set at 200 µL. Cells were incubated at 37°C, 5% CO2 for 5 days. After harvest, proliferation of CFSE-labelled responder cells was acquired by flow cytometry (FACS Calibur cytometer or on LSRFortessa™ cell analyzer) and analyzed with FlowJo software. The suppressive ability of Treg lines was assessed as the percentage of decrease in Teff proliferation in the presence of Tregs. The calculation was based on the proliferation of responder T cells alone (T_{eff} a) compared with the Teff proliferation of cultures containing a co-culture of both Teff and Treg cells (T_{eff} c) (as below).

$$\%\,\boldsymbol{Treg\ Suppression} = \mathbf{100} - \left(\left[\frac{\boldsymbol{T_{eff(c)(\%)}}}{\boldsymbol{T_{eff(a)(\%)}}}\right] \times \mathbf{100}\right)$$

2.8. *IN VITRO* REGULATORY T CELL STABILITY ASSAY

Freshly isolated, untreated and rapamycin treated $CD4^+CD25^+$ T cells (5×10^5) were activated with anti-CD3 and anti-CD28 coated beads (Dynabeads®) at 1:1 bead:cell ratio and cultured for 5 days in the presence of pro-inflammatory cytokines:

- **Mix 1**: IL-2 (10 IU/ml), IL1β (10ng/ml), IL-6 (4ng/ml) and TGF-β (5ng/ml).
- **Mix 2**: IL-2 (10 IU/ml), IL-21 (25ng/ml), IL-23 (25ng/ml) and TGF-β (5ng/ml).

Cells cultured in X vivo medium (5% HS) supplemented with IL-2 (10 IU/ml) were used as a control. At the end of the culture, cells were harvested (activation beads removed by magnetic adherence) and analysed for IL-17 expression by intracellular staining. IL-17 concentrations in supernatants following culture were analysed by an indirect sandwich ELISA.

2.8.1. INTERLEUKIN-17 ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

ELISA for human IL-17 was carried out using the Duo-Set ELISA kit. BD Falcon 96-well ELISA microplates were coated with 100 µL/well of anti-IL-17 antibody (4 µg/mL in PBS) and left overnight at room temperature. The following day plates were washed three times with 0.05% Tween 20 in PBS and blocked with 1% filtered BSA in PBS (300 µL/well) for 1 hour at room temperature. Plates were subsequently washed three times and blotted to dry. 100 µL/well of samples and standards were plated in duplicates and incubated at room temperature for two hours. The samples for IL-17 ELISA were prepared from thawed supernatants, stored at -20°C, of Tregs

cultured in IL-17 skewing conditions. The standard curve was constructed from serial two-fold dilution of manufacturer's provided standards in PBS containing 1% BSA from 1000 pg/mL to 15.63 pg/mL with one control consisting of diluent alone (0 pg/ml). The plates were washed and blotted dry three times and 100 µL of detection antibody (biotinylated anti-IL-17, 75 ng/ml) diluted in 1% BSA and PBS was added to each well and left at room temperature for two hours. Plates were washed and blotted dry three times, 100 µL of streptavidin-horse radish peroxidase (1:20 dilution in 1% BSA in PBS) was added to each well and the plates were incubated at room temperature for 20 minutes. Plates were then washed and blotted dry three times. The final step of the process required the addition of 100 µL of substrate solution to each well and incubation at room temperature for 20 minutes in the dark. The reaction was terminated with 50 µL of 2N sulphuric acid added to each well. The samples were read on an ELISA plate reader: optical density set at 450nM. The relative concentration of IL-17 in each sample was calculated from the standard curve, consisting of optical densities of known concentrations and reported relative per $1x10^6$ cells.

2.9. Heme-oxygenase (HO-1) Enzyme Linked ImmunoSorbent Assay (ELISA)

ELISA for human HO-1 was carried out using the ImmunoSet™ HO-1 (human), ELISA development kit. HO-1 Capture antibody (3.5 µg/ml) was diluted (1:250) in coating buffer (10 mM sodium phosphate, 15 mM NaCl, pH 7.4) and plated on to a BD Falcon 96-well ELISA Microplate (100 µl/well) then the plate sealed and incubated overnight at room temperature. The wells were then aspirated and blocked

with 200 µl/well of 10 mM sodium phosphate, 15 mM NaCl, 1.0% BSA, 1.0% sucrose, pH 7.4 and the plates sealed and incubated at room temperature for at least 1 hour.

The samples for HO-1 ELISA were prepared from snap frozen cell pellets of regulatory T cells stored at -80^{o}C and were thawed on ice. RIPA Cell Lysis Buffer 2 containing a protease inhibitor cocktail (1:200 dilution) was added to the cell pellet to achieve a concentration of 4 x 10^{6} cells/ml. The cell pellet was resuspended by gentle pipetting and homogenized by passing through a 25G needle. The suspension was incubated on ice for 30 minutes and mixed intermittently. Following this the suspension was centrifuged at 12000 rpm for 10 minutes in a 4°C refrigerated microfuge. The supernatants (cell lysate) were assayed immediately.

The plates were aspirated so as to remove the blocking solution. Subsequently, 100 µl/well of standard and 100 µl/well of sample were added in duplicates and the plates sealed and incubated at room temperature for 1 hour. Standard curves were generated using the following concentrations of recombinant HO-1: 12.5 ng/ml, 6.25 ng/ml, 3.12 ng/ml, 1.56 ng/ml, 0.78 ng/ml, 0.39 ng/ml, 0.19 ng/ml, 0.097 ng/ml, 0.049 ng/ml, 0.025 ng/ml and 0 ng/ml diluted in in 100 mM sodium phosphate, 150 mM NaCl, 1.0% BSA, 0.1% Tween-20. The wells were then aspirated and washed with 400 µl/well of wash buffer (10 mM sodium phosphate, 15 mM NaCl, 0.1% Tween-20) four times. Detection antibody was diluted (1:250) in 100 mM sodium phosphate, 150 mM NaCl, 1.0% BSA, 0.1% Tween-20, and 100 µl/well added to each well (except to the well containing diluent alone). The plates were sealed and incubated for 1 hour at room temperature. The wells were then aspirated and washed with 400 µl/well of wash buffer four times. 100 µl/well Streptavidin-horseradish peroxidase at 1:600 dilution in 100 mM sodium phosphate, 150 mM NaCl, 1.0%, BSA, 0.1% Tween-20,

were then added and the plates sealed and incubated for 30 minutes at room temperature. The wells were then aspirated and washed with 400 µl/well of wash buffer four times. 100 µl/well of substrate solution consisting of TMB and stabilized hydrogen peroxide were then added and the plates incubated for a maximum of 30 minutes in the dark at room temperature. 100 µl/well of 1M Hydrochloric acid was then added to stop the reaction. The plates were read using an ELISA reader at 450 nm within 30 minutes. HO-1 concentration in the samples was determined by interpolation from the standard curve.

2.10. STATISTICAL ANALYSIS

Statistical analysis was carried out on GraphPad Prism 5.0c (GraphPad software Inc., USA). Parametric and nonparametric data were expressed as mean ± standard error. For comparison of parametric data, which were normally distributed, paired and unpaired students t-tests were used, for linked and unlinked data respectively. Statistical significance was set at $p < 0.05$. *P<0.05, ** P<0.01 and *** P<0.001.

Chapter 3

An In-depth Phenotypic and Functional Characterisation of Regulatory T Cells from Patients with Alcohol-related Cirrhosis

3.1. Introduction and Objectives

The quintessence of clinical trials of adoptive Treg cell therapy, such as the ThRIL study, rests on a sure foundation of pre-clinical studies demonstrating that Tregs from liver transplant recipients can be isolated and expanded, reaching numbers suitable for their clinical translation, whilst maintaining their phenotype and function throughout. Moreover, since ARC is the primary indication for liver transplantation in the UK, this patient cohort will form the majority of subjects recruited into the ThRIL study. As such it is fundamentally important to firstly carry out an in-depth characterisation of the Tregs from patients with ARC prior to their *ex vivo* expansion.

The pertinence of such an endeavor is also supported by studies showing a quantitative deficiency in Treg numbers in several liver diseases, including autoimmune hepatitis (Longhi et al. 2005), viral hepatitis (Xu et al. 2006) and primary biliary cirrhosis (Lan et al. 2006). Moreover, a defect in Treg function is also well documented in a number of liver diseases (Lan et al. 2006, Longhi et al. 2006, Sebode et al. 2014). To date, there have not been any studies on Tregs isolated from patients with ARC and, bearing in mind the varied similarities in immune dysfunction seen in liver diseases, such defects in Treg numbers and function may also become apparent upon investigation in this patient cohort. Moreover, and as already eluded to, research on immunological changes have provided abundant data to support the notion that immune-mediated injury plays a major role in the development, perpetuation and outcome of alcohol related liver disease (Laso et al. 1999, Gao et al. 2011, Albano 2012).

This chapter hypothesizes that there is a defect in Treg number and function in patients with ARC.

The basis of this hypothesis is not only founded on the studies outlined above, but also in view of the environment that the cells are exposed to *in vivo* and its potential downstream effect on Treg phenotype and function. In this regard, accumulating evidence suggests that liver cirrhosis is associated with chronic low-grade inflammation. Moreover, studies have shown a positive correlation between disease severity in cirrhosis and the presence of advanced oxidation protein products (AOPPs) (Zuwala-Jagiello et al. 2011), protein markers of oxidative stress with proinflammatory properties, which are known to further fuel this inflammatory microenvironment.

Addressing the following aims will test the hypothesis stated for this chapter:

1. Isolation of Tregs from patients with ARC with comparison of Treg numbers and phenotype to age and sex matched healthy controls.
2. Assessment of Treg function.
3. Assessment of the influence of a proinflammatory milieu on the Treg stability.
4. Investigation into the role of heme oxygenase-1 (HO-1) in Treg mediated immune suppression.

3.2. PATIENTS WITH ALCOHOL RELATED CIRRHOSIS HAVE A LOWER FREQUENCY OF $CD4^+CD25^+CD127^{LO}$ REGULATORY T CELLS AS COMPARED TO HEALTHY CONTROLS

There have already been reports of decreased numbers of circulating $CD4^+CD25^{hi}$ Tregs in various autoimmune liver diseases (Longhi et al. 2005, Lan et al. 2006, Sebode et al. 2014). In view of reports of the similarities between liver disease secondary to alcohol and autoimmune liver disease (Perperas et al. 1981, Chedid et al. 1994, Ma et al. 1997). I next sought to investigate the relative number of circulating Tregs in ARC patients.

To compare the percentage of circulating Tregs between ARC patients and age and sex matched HCs, enriched CD4+ T cells were phenotyped by flow cytometry. The percentage of $CD25^{Hi}$ in the total CD4+ population did not differ between the two cohorts (4.13% ± 0.932 as compared to 4.31% ± 0.889 for ARC patients and HCs, respectively, p=0.889) **(Figure 3.1).**

Of note, however, several subsets of Tregs have been described to date, with reports that $CD4^+CD25^{hi}$ $FOXP3^+$ Tregs typically lack the expression of the interleukin (IL)-7 receptor α chain, CD127 (Liu et al. 2006). The differential expression of CD127 has been used to denote an optimally pure population of Tregs that is inversely correlated with FOXP3 levels and the suppressive function of human $CD4^+$ Tregs. As such a further in-depth phenotypic analysis was carried out to compare levels of $CD4^+CD25^+CD127^{Lo}$ Tregs between ARC and HCs. The data revealed that patients with ARC have a decreased frequency of $CD4^+CD25^+CD127^{Lo}$Tregs as compared to HCs (p=0.0001) **(Figure 3.2),** mirroring the results seen in autoimmune hepatitis**.**

This data may provide further support to the notion that immune-mediated injury plays a role in the development and perpetuation of this condition (Laso et al. 1997, Lin et al. 2008, Gao et al. 2011, Albano 2012).

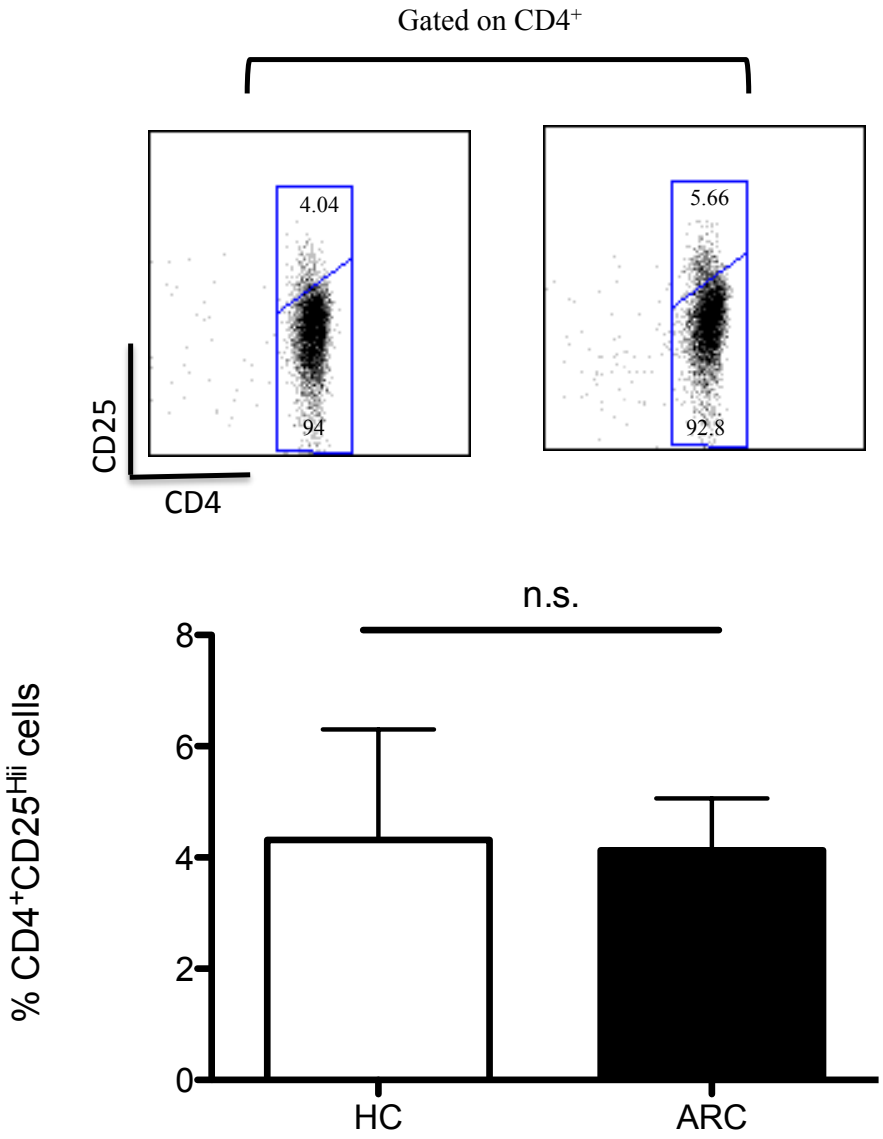


FIGURE 3.1. PERCENTAGE OF CIRCULATING $CD4^{+}CD25^{HI}$ TREGS

Representative dot plot and graph denoting the circulating percentage of $CD4^{+}CD25^{Hi}$ Tregs of 5 ARC patients and 5 HCs. n.s.: not significant. Error bars represent SEM.

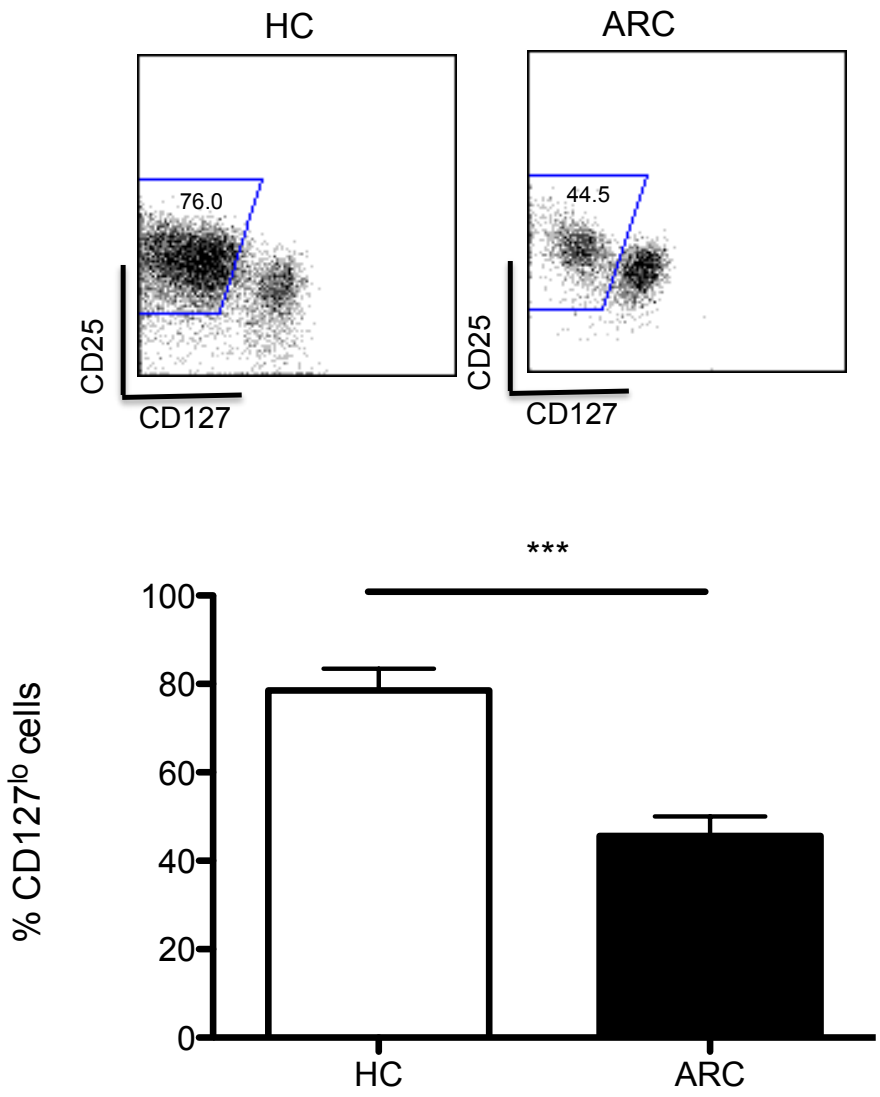


FIGURE 3.2. FREQUENCY OF $CD4^{+}CD25^{+}CD127^{LO}$ TREGS.

Representative dot plot and graph denoting the relative frequency of $CD4^{+}CD25^{+}CD127^{Lo}$ Tregs from 20 HCs and 20 ARC patients on isolation. . *** p=<0.001. Error bars represent SEM.

3.3. A DEFECTIVE REGULATORY T CELL SUPPRESSOR FUNCTION IN PATIENTS WITH ALCOHOL RELATED CIRRHOSIS AS COMPARED TO HEALTHY CONTROLS

Alongside a deficiency in circulating Treg numbers seen in autoimmune liver diseases, it has also been noted that the Tregs *in vivo* display defective suppressor profiles (Lan et al. 2006, Longhi et al. 2006, Sebode et al. 2014). However, to date there are no studies on the functional assessment of Tregs from patients with ARC. As such, the suppressive function of 20 ARC patients and 20 age and sex matched HCs was compared.

A CFSE dilution assay was performed to assess the ability of freshly isolated Tregs from ARC patients and HCs to suppress the proliferation of allogeneic Teffs **(Figure 3.3A).** Freshly isolated ARC patient Tregs were found to possess an impaired capacity of suppression of Teff proliferation as compared to HCs at different Treg:Teff ratios (ARC vs HC: **1:1** 19.6% ± 3.33 vs 72.7% ± 2.54, p <0.0001; **1:5** 13.3% ± 3.93 vs 40.6% ± 3.45, p <0.0001; **1:10** 10.3 ± 3.19 vs 30.67± 3.65, p= 0.0002 **(Figure 3.3A).** This is the first report documenting these findings that further directed the rest of the study.

ARC is the end product of a multi-step process initiated by excessive alcohol consumption. In view of the well-documented immunomodulatory roles of alcohol, we next investigated whether the evident lack of Treg suppressive function was attributable to a history of chronic alcohol consumption effects or the general state of cirrhosis. In this regard, the suppressive function of Tregs isolated from ARC patients was compared to Tregs from Non- Alcoholic- Steatohepatitis (NASH) patients, where alcohol plays no role in the development of cirrhosis. The data clearly demonstrated

that the suppressive function of Tregs from both cohorts of patients was comparably low, suggestive that the current state of cirrhosis, common to both cohorts of patients, was linked with the Treg dysfunction as opposed to historic alcohol consumption in ARC patients **(Figure 3.3B).**

I next sought to uncover any possible correlation between this novel discovery and each patient's clinical history. Interestingly and of irrefutable significance was the association between the severity of the liver disease, as determined by the Model for End-stage Liver Disease (MELD) score, and Treg suppressor function **(Figure 3.4A).** However, upon dissection of the MELD score into its various components (bilirubin, INR and creatinine) there was no correlation between Treg function and each constituent in isolation **(Figure 3.4B**), suggesting that a combination of all parameters, relating to the overall severity of liver disease, was directly associated with the Treg dysfunction.

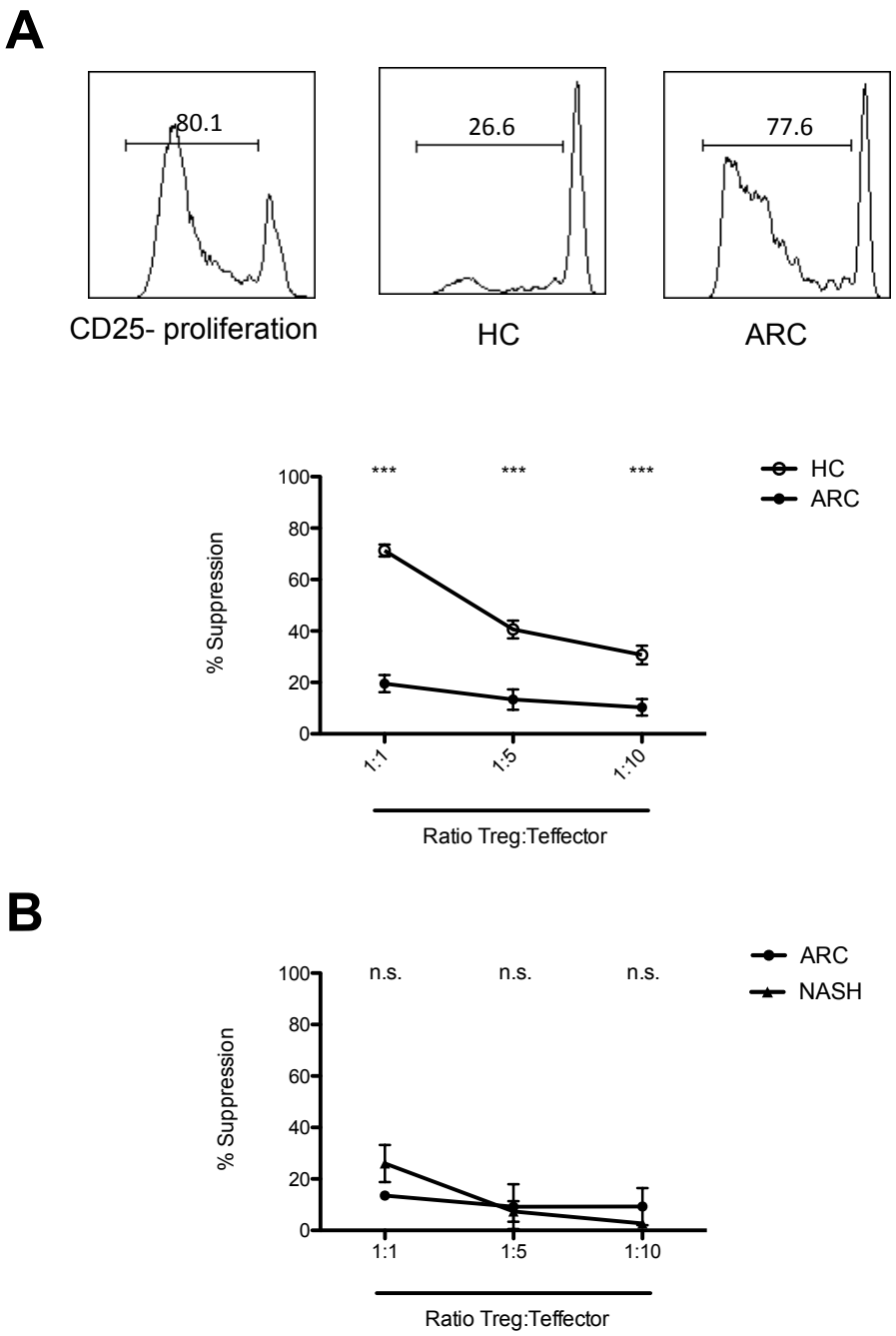


FIGURE 3.3. ASSESSMENT OF TREG SUPPRESSOR FUNCTION

A. Representative histogram and graph displaying the suppressor function of freshly isolated Tregs from 20 HCs and 20 ARC patients. CFSE dilution assay was conducted and 3 different Treg:Teff ratios: 1:1, 1:5 and 1:10 investigated **B**. Treg Suppressor function of 10 patients with cirrhosis, 5 ARC and 5 NASH, was compared at different ratios of Treg:Teff. *** p=<0.001. *Abbreviation: n.s- not significant. Error bars represent SEM.*

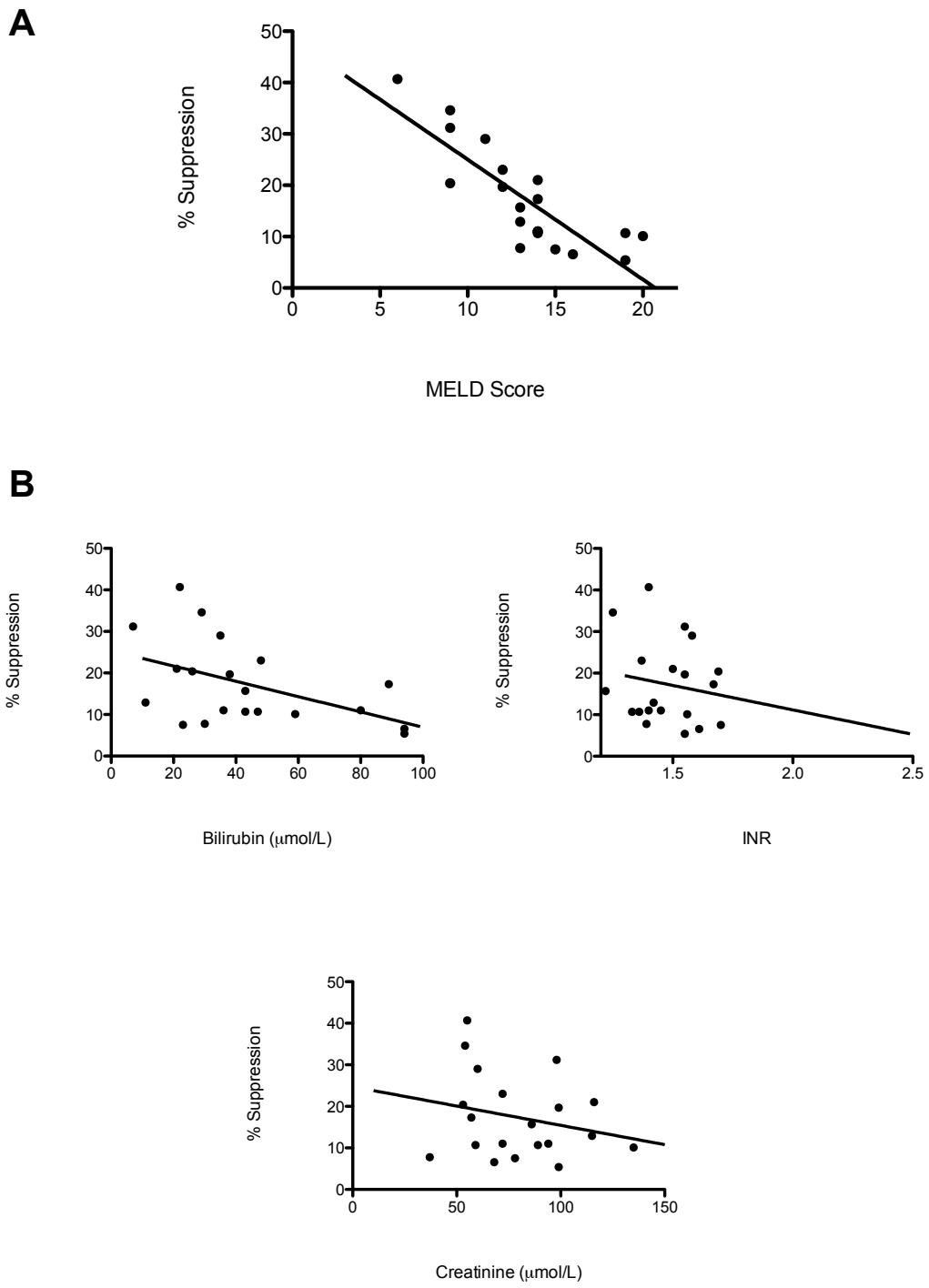


FIGURE 3.4. CORRELATION OF TREG SUPPRESSOR FUNCTION WITH SEVERITY OF LIVER DISEASE

A. Graph correlating Treg suppressor function and the MELD score of 20 ARC patients **B.** Graphs depicting each variant of the MELD score (Bilirubin, INR, Creatinine) against Treg suppressor function of 20 ARC patients. *Abbreviations; MELD- model for end stage liver disease, INR- international normalized ratio.*

3.4. Regulatory T cells from patients with alcohol related cirrhosis express lower levels of CTLA-4, CD62L/CD27 and Helios, with similar levels of activation/maturation markers as compared to Healthy controls

Human Tregs are known to express a wide array of different markers dependent on their distinct immunological role *in vivo*. Therefore, Tregs from both ARC patients and HCs were next extensively characterized in order to unmask an explanation for this apparent lack of suppressive function.

A constitutive high expression of the cytotoxic T lymphocyte antigen 4 (CTLA-4) represents a well-documented component of $CD4^+CD25^+$ Tregs that has also been shown to contribute to their suppressive function (Annunziato et al. 2002, Manzotti et al. 2002). Analysis of CTLA-4 expression in Tregs from ARC patients revealed a significant decrease in the percentage of $CD4^+CD25^+CTLA4^+$ cells, as compared to HCs, 12.6% ± 2.63 vs 25.3% ± 1.71, respectively p=0.0008 **(Figure 3.5A).**

On a similar note, the co-expression of CD62L and CD27 on Tregs has been reported to denote a Treg population with high suppressive capabilities both *in vitro* and *in vivo* (Koenen et al. 2005, Koenen et al. 2008, Issa et al. 2010, Nadig et al. 2010). The co-expression of these two markers on ARC Tregs was again significantly lower 68.3% ± 3.38 as compared to HCs 81.0% ± 1.91, p= 0.0043 **(Figure 3.5A).**

Helios, the intracellular Ikaros transcription factor has been thought to delineate a population of thymic derived naturally occurring Tregs (Thornton et al. 2010) with enhanced regulatory potential (Zabransky et al. 2012). Upon investigation of this marker, a lower percentage of Helios^{+} Tregs from ARC patients 43.7% ± 3.42 as compared to HCs 61.3% ± 4.18, p=0.0044 was determined, suggesting a paucity of naturally occurring Tregs in ARC patients. However, it is pertinent to note that the use of this marker to identify thymic derived naturally occurring Tregs is controversial and has come under great scrutiny (Getnet et al. 2010, Himmel et al. 2013, Edozie et al. 2014).

To evaluate whether differences existed in Treg activation and maturation markers between HCs and ARC patients, the percentage of Tregs expressing the ectoenzyme, CD39, and HLA-DR was investigated in both cohorts. Similar expression of both markers was reported following phenotypic analysis of Tregs from ARC patients as compared to HCs, CD39, p=0.193 and HLADR, p=0.179 **(Figure 3.5A).**

Additionally, the expression of CXCR3, the chemokine receptor associated with Treg homing and migration to the liver (Oo et al. 2010) was analysed showing that a similar percentage of Tregs from both cohorts express this marker ARC 80.6% ± 3.74 vs HC 82.7% ± 1.91, p=0.632.

Further in-depth characterisation of the Tregs from ARC patients was carried out **(Figure 3.5B),** investigating a panel of key markers known to be employed by Tregs

to exert their regulatory role, namely T cell immunoglobulin mucin 3 (TIM3) (Sakuishi et al. 2013), FASL (Gorbachev and Fairchild 2010), granzyme B (Loebbermann et al. 2012), PD1 and PDL1 (Jacobs et al. 2009), GITR (McHugh et al. 2002), ICOS and ICOSL (Busse et al. 2012) and GARP (Stockis et al. 2009). However, FACS analysis revealed no differences in the expression of these markers by Tregs between the two cohorts that could potentially account for the lack of Treg suppressive function seen in ARC patients **(Figure 3.5B).**

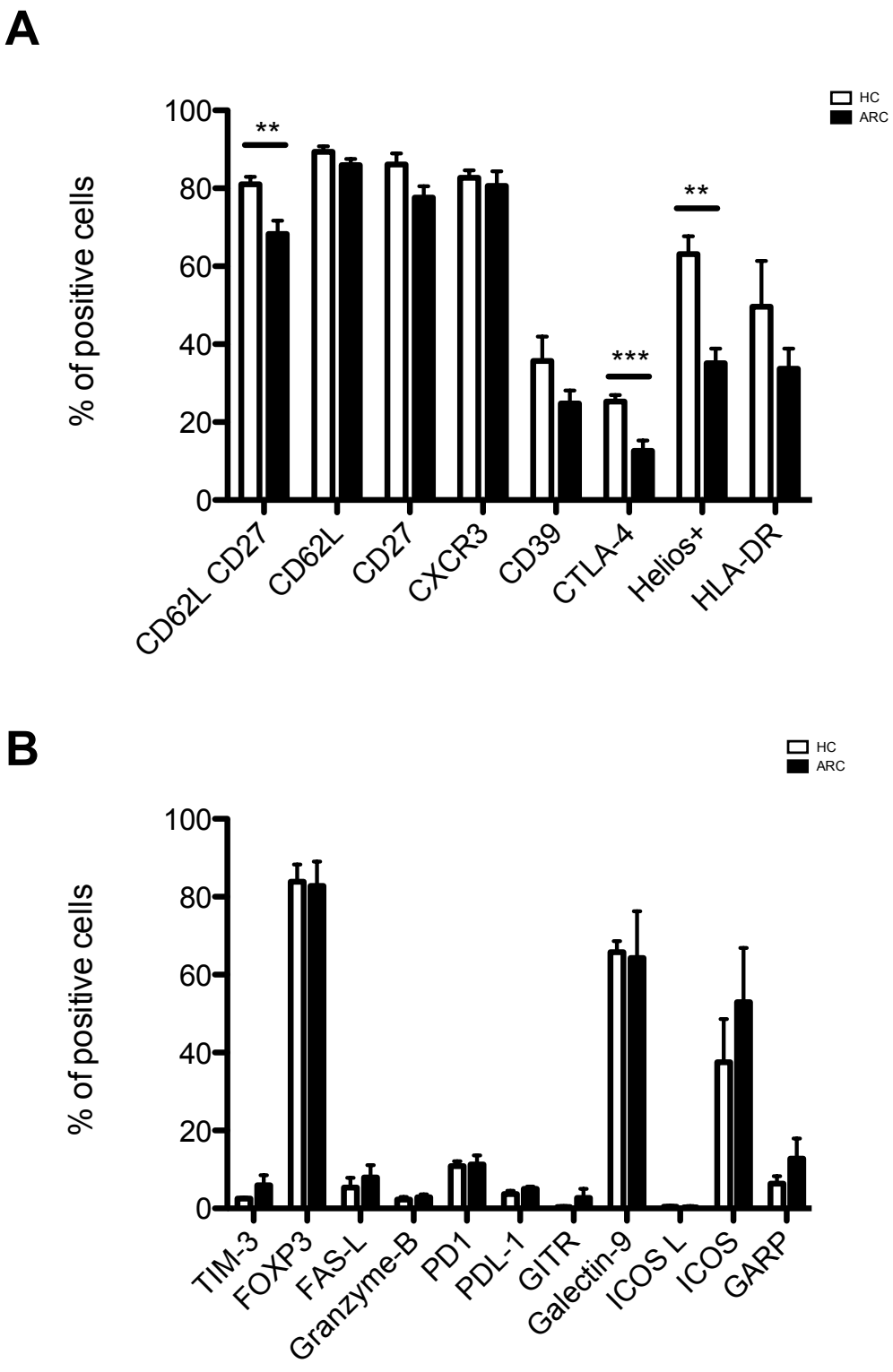


FIGURE 3.5. EXPRESSION OF DIFFERENT PHENOTYPIC AND FUNCTIONAL TREG MARKERS.

A. Pooled data from 10 ARC patients and 10 age and sex matched HCs comparing the mean percentage expression of each marker on $CD4^{+}CD25^{+}$ Tregs. **B.** Pooled data from 10 ARC patients and 10 HCs comparing the mean percentage of various functional markers on $CD4^{+}CD25^{+}$ Tregs. ** p=<0.01 and *** p=<0.001. Error bars represent SEM.

3.5. PATIENTS WITH ALCOHOL RELATED CIRRHOSIS HAVE A SIMILAR PERCENTAGE AND DISTRIBUTION OF MARKERS IN REGULATORY T CELL SUBPOPULATIONS AS COMPARED TO HEALTHY CONTROLS.

Human Tregs have been defined as a heterogeneous population of cells that can be divided into three phenotypically and functionally distinct sub-populations based on the differential expression of the naïve cell marker CD45RA and the level of FOXP3 expression (Miyara et al. 2009). **Figure 3.6A** depicts the gating strategy used to delineate the human Treg compartment into population I; naïve or resting Tregs ($CD45RA^{+}FOXP3^{Lo}$), population II; effector Tregs ($CD45RA^{-}FOXP3^{Hi}$), both of which are suppressive in vitro, and population III; the non-suppressive, cytokine secreting non-Tregs ($CD45RA^{-}FOXP3^{Lo}$). As such and to determine whether there were differences in the Treg subpopulations that could potentially explain the lack of Treg suppressor function in patients with ARC, the percentages of each of the three populations was compared between ARC patients and HCs. Population III was the largest subpopulation from ARC patients 42.7% ± 3.05, this was comparable to HCs 53.1% ± 5.17, p=0.101. In addition, similar proportions of Treg subpopulations I and II was reported between the two groups **(Figure 3.6B)**.

Moreover, the expression of various markers of activation/maturation was also assessed on each of the defined subpopulations. In both cohorts, a step-wise increase in the percentage of cells expressing CD39 and HLA-DR was detected between population I, III and II, correlating with the maturation status. Expression of these markers on Treg subpopulations did not, however, differ between ARC patients and HCs (**Figure 3.6C, 3.6D)**.

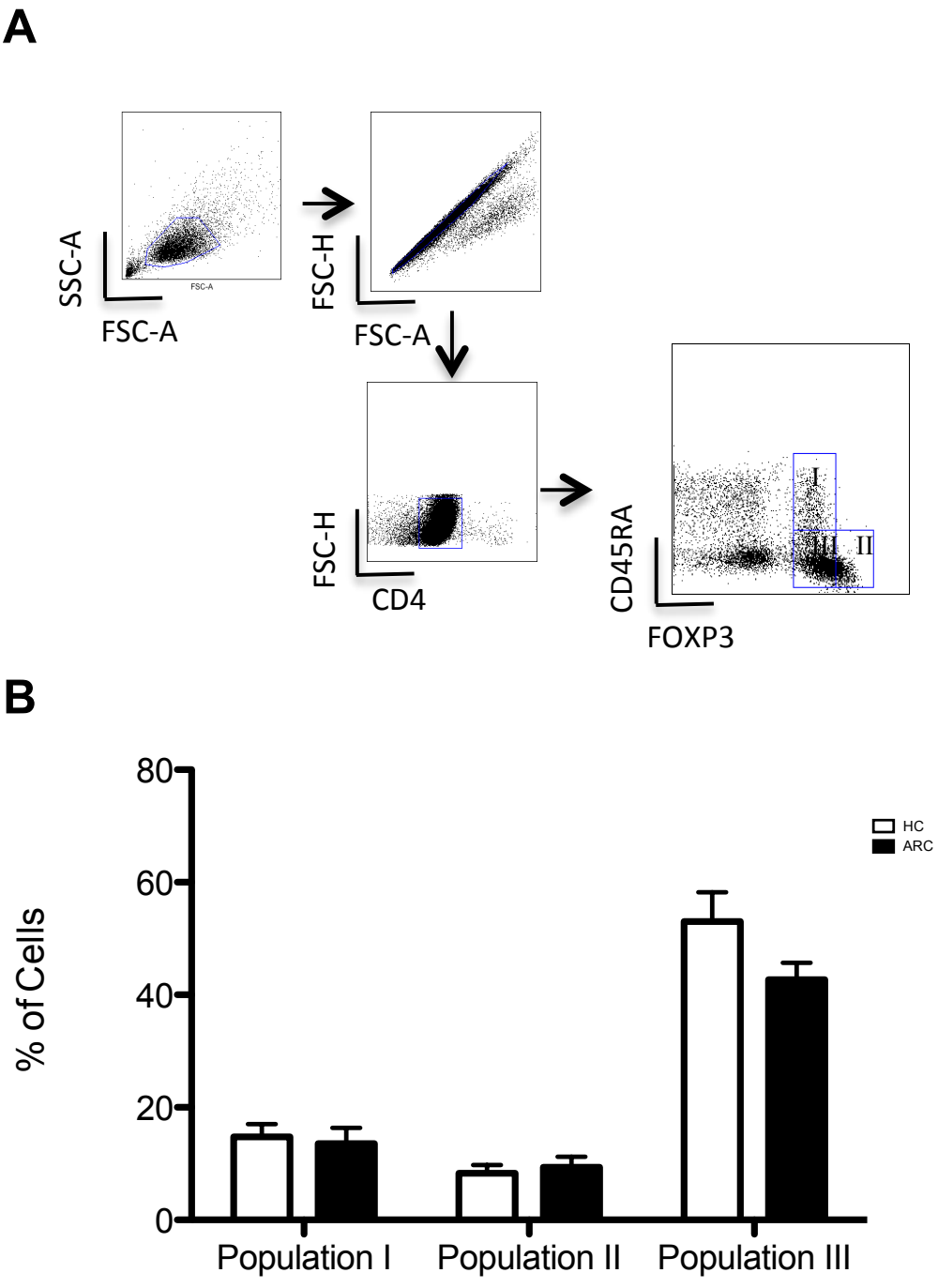


FIGURE 3.6. PHENOTYPIC ANALYSIS BASED ON TREG SUB-POPULATIONS.

A. Gating strategy used to divide Foxp3^{+}tregs into the three sub-populations adopted from Miyara et al. 2009 (population I: CD4^{+}CD45RA^{+}Foxp3^{lo}, population II: CD4^{+}CD45RA^{-} Foxp3^{Hi}, population III: CD4^{+}CD45RA^{-}Foxp3^{lo}). B. Graph depicting mean percentage of each Treg subpopulation (I-III) from 10 HCs and 10 ARC patients. . *p=<0.05,** p=<0.01 and *** p=<0.001. Error bars represent SEM

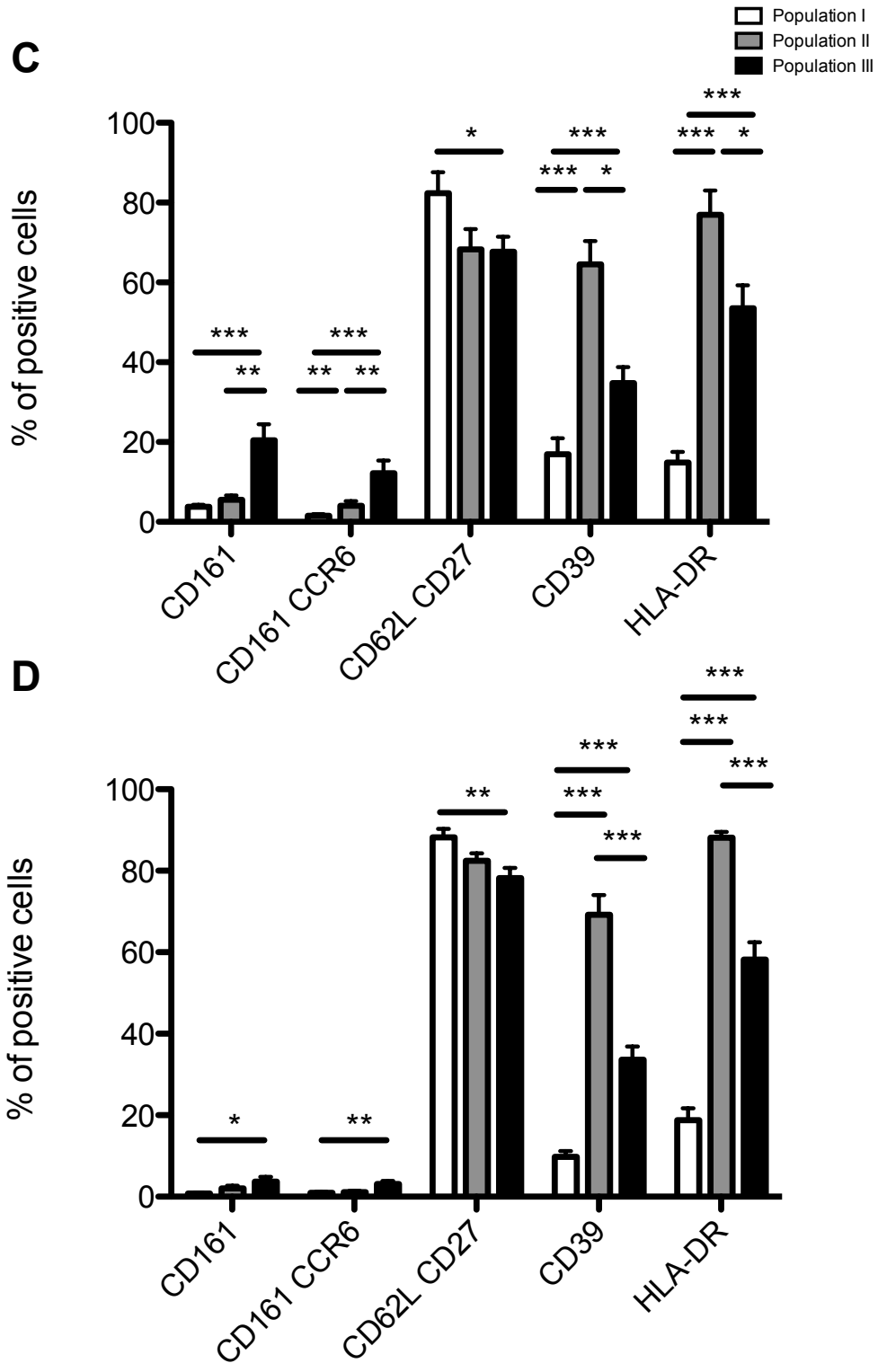


FIGURE 3.6. PHENOTYPIC ANALYSIS BASED ON TREG SUB-POPULATIONS.

C. Graph of pooled data from 10 ARC patients showing the mean percentage expression of Treg surface markers based on each of the three gated subpopulations. **D.** Graph of pooled data from 10 HCs showing the mean percentage expression of Treg surface markers on the three sub-populations. *p=<0.05,** p=<0.01 and *** p=<0.001. Error bars represent SEM.

3.6. Regulatory T cells from patients with alcohol related produce Interleukin-17 and Interferon-γ in the presence of pro-inflammatory cytokines and express high levels of CD161

The plasticity of Tregs has been well documented with studies suggesting that Tregs can convert to cells producing inflammatory cytokines particularly when exposed to a pro-inflammatory milieu (Hori 2010). To assess the stability of Tregs from ARC patients in comparison to HCs, freshly isolated Tregs from both cohorts were cultured for 5 days in the presence of pro-inflammatory cytokines (described in materials and methods) and the percentage of $IL17^+$ and IFN-γ^+ cells analysed by FACS. As compared to HCs, there was an increase in the percentage of $FOXP3^+IL17^+$ and $FOXP3^+$ IFN-γ^+ cells when ARC patient Tregs were cultured in the presence of pro-inflammatory cytokines (**$FOXP3^+IL17^+$; Mix 1** ARC 5.86% ± 0.888 vs HC 1.45% ± 0.324, p= 0.0002; **Mix 2** 4.79% ± 1.01 vs 1.45% ± 0.324, p= 0.0056 and **$FOXP3^+$ IFN-γ^+**; **Mix 1** ARC 6.84% ± 0.908 vs HC 3.86% ± 0.569, p= 0.0124; **Mix 2** 5.25% ± 0.644 vs 4.40% ± 0.815 p= 0.424) (**Figure 3.7A, 3.7B)**.

More recently my group and another, have identified a subpopulation of Tregs within population III that is responsible for the production of IL-17 by Tregs (Afzali et al. 2013, Pesenacker et al. 2013). This marker was initially shown to be expressed by Th17 cells (Cosmi et al. 2008, Maggi et al. 2010). The expression of CD161 on the Tregs from ARC patients and HCs was investigated. We show that as compared to HC Tregs, 5.48% ± 1.07, there is an increased percentage of $CD161^+$ Tregs isolated

from ARC patients 18.0% ± 1.94, p<0.0001 (**Figure 3.7C**), which, upon further delineation, is found to be mostly expressed on population III (**Figure 3.6C**).

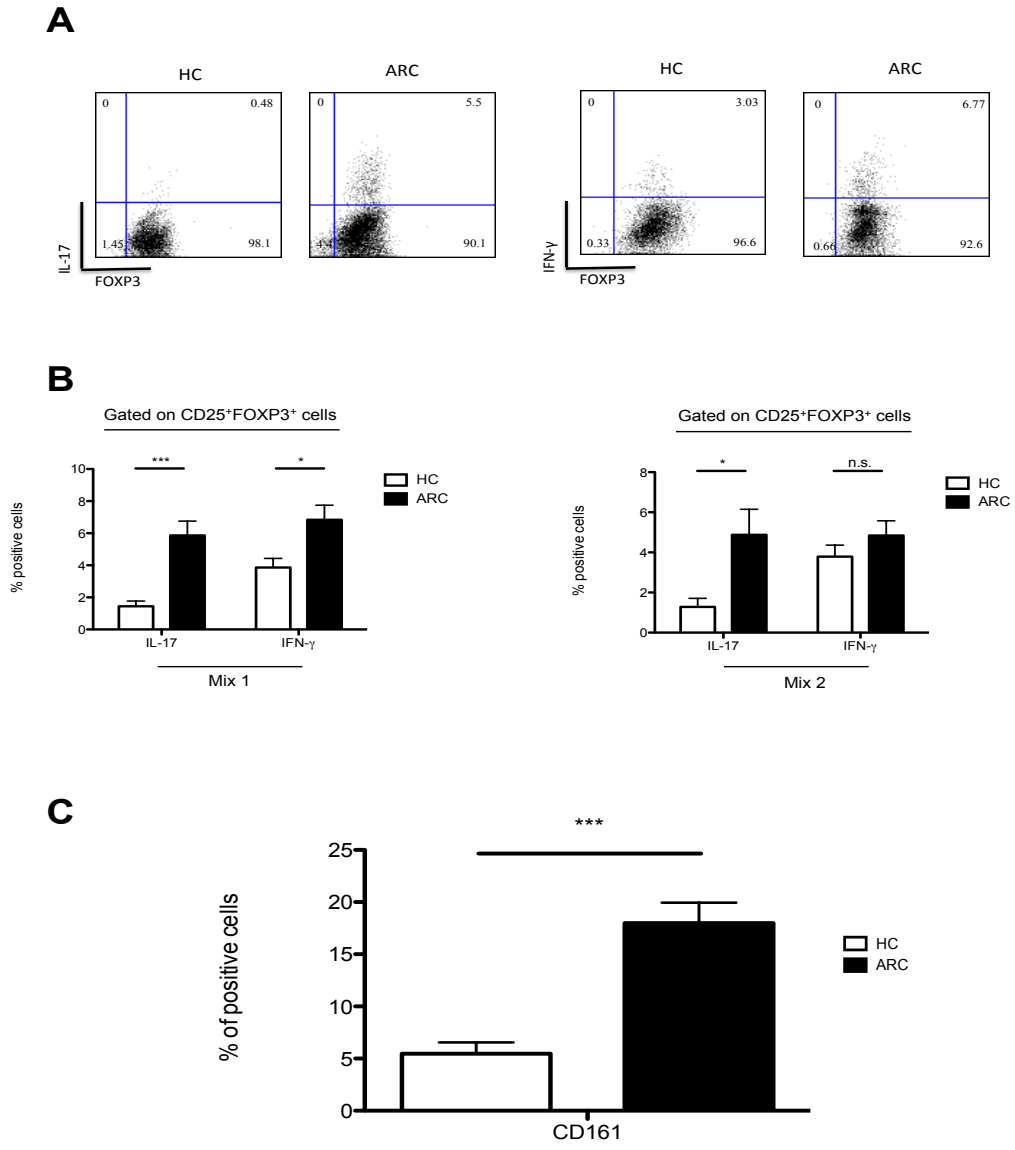


FIGURE 3.7. ASSESMENT OF TREG STABILITY.

A. Representative dot plots from 10 HCs and 10 ARC patients depicting the gating of FOXP3$^+$IL-17$^+$ and FOXP3$^+$IFN-γ$^+$ Tregs. **B.** Graph showing the frequency of IL-17$^+$ and IFN-γ$^+$ Tregs from 10 HCs and 10 ARC patients following 5 day cultures with pro-inflammatory cytokines (**Mix 1**- IL-2, IL1β, IL-6 and TGF-β and **Mix 2:** IL-2, IL-21, IL-23 and TGF-β). **C.** Graph comparing the mean percentage of freshly isolated CD4$^+$CD25$^+$ Tregs expressing CD161 from 10 HCs and 10 ARC patients. ** p=<0.01 and *** p=<0.001. Error bars represent SEM.

In line with these findings, and as eluded to earlier, accumulating evidence suggests that liver cirrhosis is associated with a chronic state of low-grade inflammation (Giron-Gonzalez et al. 2004). This supports the data presented, whereby the environment the Tregs are exposed to *in vivo* during cirrhosis, may account for their plasticity and propensity to convert to IL-17^{+} and IFNγ^{+} cells, perpetuating the inflammatory state.

3.7. Regulatory T cells and Heme Oxygenase-1

3.7.1. Regulatory T cells from patients with alcohol related cirrhosis exhibit lower levels of Heme Oxygenase-1 as compared to Healthy Controls

It has been reported that the inflammation that characterises liver cirrhosis is often caused by a rise in free radicals within the liver (Kirkham 2007, Videla 2009). Under normal circumstances, the liver maintains a supply of internal anti-oxidants to neutralize the free radicals generated by various endo and exogenous compounds processed in the liver. However, when the liver is exposed to continuous oxidative insults i.e. long lasting alcohol abuse, the damage from free radicals increases, resulting in inflammation and the formation of fibrosis (Czeczot et al. 2006, Valko et al. 2007). Moreover, several lines of evidence provide a link between the oxidative stress and the inflammatory state during cirrhosis and suggest that advanced oxidation protein products, present in the plasma of cirrhotic patients act as inflammatory mediators. In addition, and more importantly, a positive correlation between severity

of liver disease and the presence of such products of oxidative stress has been reported, with a further correlation with the inflammatory markers (Zuwala-Jagiello et al. 2009, Zuwala-Jagiello et al. 2011). Based on our finding of a Treg dysfunction in patients with ARC and its association with liver disease severity, the role of the anti-oxidant pathway in this system was next investigated.

In view of the wealth of data on the anti-inflammatory (Otterbein et al. 2000), anti-oxidative (Yachie et al. 1999) and antiapoptotic (Ke et al. 2002) role of HO-1 and studies to suggest that Tregs also express HO-1, I next sought to determine whether a defect in the expression of this enzyme by Tregs isolated from ARC patients could contribute to the Treg dysfunction seen in this condition.

As such, quantitative determination of HO-1 was carried out from Treg cell lysates of ARC patients and HCs, using ELISA. The data clearly demonstrated that freshly isolated Tregs, obtained from ARC patients, have decreased levels of HO-1, $p= 0.0271$ **Figure 3.8A.** This finding was also confined to Tregs, whereby measurement of HO-1 levels in $CD4^{+}CD25^{-}$ effectors T cells, from the same donor as the Tregs, showed similar levels of HO-1 as compared to age and sex matched HCs $CD4^{+}CD25^{-}$ **Figure 3.8B.**

3.7.2. Inhibition of Heme Oxygenase-1 activity by Zinc Protoporphyrin results in decrease Treg suppressor function.

In order to confirm the role of HO-1 in Treg suppressor function, a specific competitive inhibitor of HO-1 activity, zinc protoporphyrin (ZnPP), was used. Studies have shown that ZnPP irreversibly binds and inactivates HO-1 enzymatic activity (Kappas and Drummond 1986) **(Figure 3.8C).**

Tregs from 5 healthy donors were isolated and cultured for 24 hours in the presence of the inhibitor with subsequent analysis of suppressive function.

The data obtained demonstrated that HO-1 plays an important role in the suppressive function of Tregs. It was shown that the inhibition of HO-1 by ZnPP resulted in a decreased Treg suppressive function, as compared to untreated cultures, and that this effect was dose dependent (Treg suppression of Teff proliferation at Treg:Teff 1:1, untreated cultures; 43.3% ± 7.67; 25μM ZnPP treatment; 24.5% ± 7.33 and 50μM ZnPP treatment; 21.7% ± 4.34, Untreated vs ZnPP 50μM, p= 0.0281)

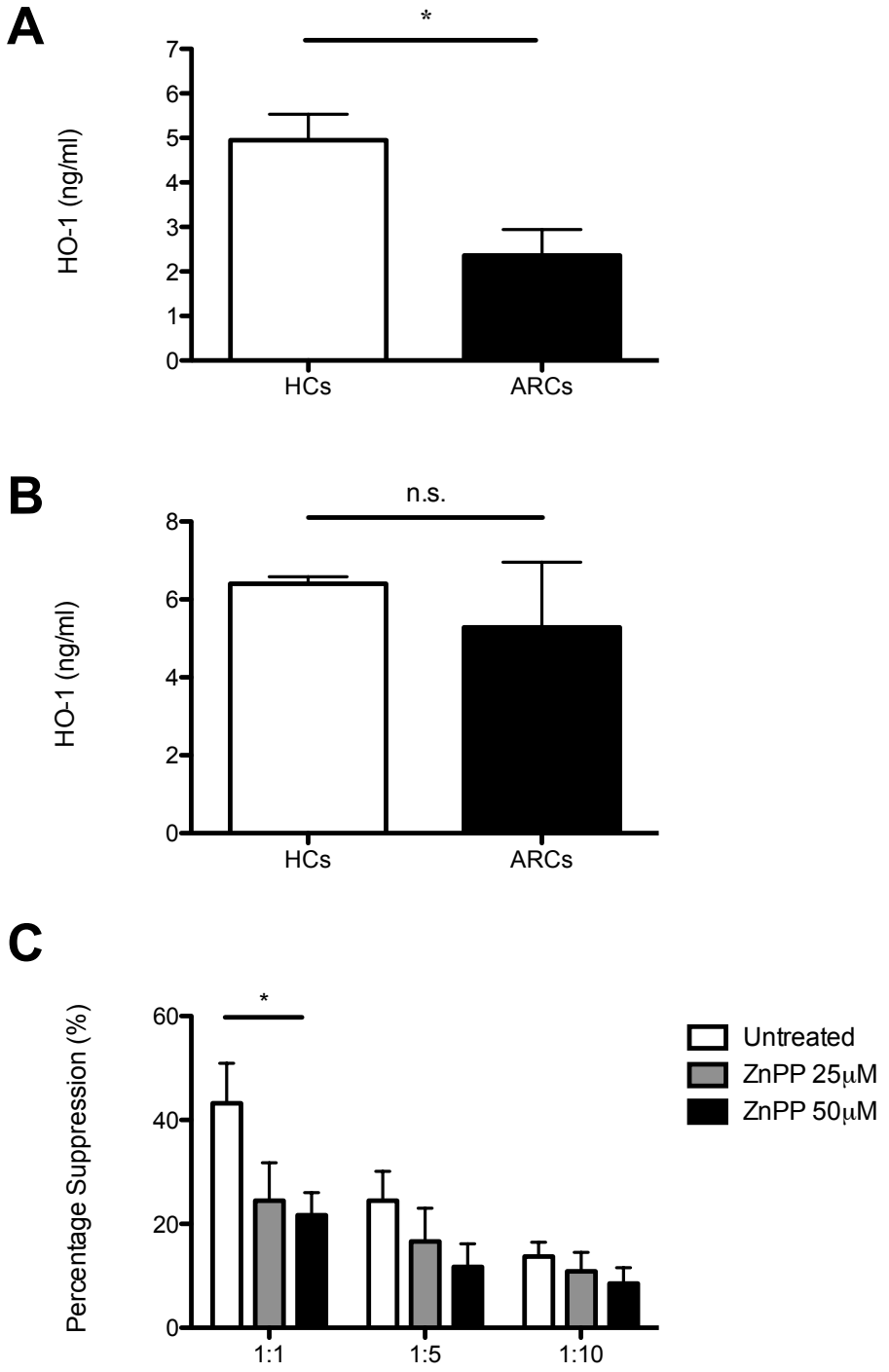


FIGURE 3.8. DIFFERENTIAL EXPRESSION OF HO-1 BY T CELL POPULATIONS.

A. Graph comparing the relative expression of HO-1 by $CD4^{+}CD25^{+}$ Tregs**. B.** Graph comparing the relative expression of HO-1 by $CD4^{+}CD25^{-}$ Teffector cells**. C.** Graph of 5 HCs showing Treg suppressor function following 24 hours of culture in the presence (25μM and 50μM) and absence of the HO-1 inhibitor, ZnPP. Suppressor function was assessed, using a CFSE dilution assay at 3 different Treg:Teff ratios.1:1, 1:5 and 1:10. *p=<0.05. Error bars represent SEM. *Abbreviations; Heme Oxygenase-1 (HO-1), Zinc protoporphyrin (ZnPP).*

3.8. DISCUSSION

There has been a global rise in mortality due to alcohol related liver disease in the western world, with ARC being a major indication for transplantation (Seth et al. 2011). In view of the shortage of donor organs and the several challenges currently facing liver transplantation, there is undoubtedly a pressing need to find novel modalities of treatment for tolerance induction in the setting of transplantation, with Treg therapy being at the forefront of research.

The first key steps in using autologous Tregs as treatment modality for tolerance induction rely solely on understanding the biology of Tregs from patients who will be receiving the therapy. In this regard, this chapter summarises the data on the in-depth phenotypic and functional characterisation of Tregs isolated from patients with ARC with direct comparison with age and sex matched HCs.

A decrease in frequency of Tregs expressing $CD127^{lo}$ was evident from ARC patients when compared to HCs. Moreover, and of importance was the novel finding of a defect in Treg suppressor function in patients with ARC and its important correlation with disease severity, assessed by the MELD score **(Figure 3.4A).** In line with these findings Almeida et al. have reported decreased number of peripheral blood Tregs in patients with alcoholic hepatitis, whilst normal numbers of these cells were reported in chronic alcoholics who had no signs of liver disease (Almeida et al. 2013). Such data may suggest that in patients with liver disease and/or cirrhosis, the decrease in Treg numbers could result in the perpetuation of an on-going inflammatory state, aggravating the disease progression.

In agreement, the partial penetrance of ARC whereby some, but not all, alcoholics develop cirrhosis alludes that other cofactors besides direct toxicity of alcohol are at work in determining an individual's predisposition to developing ARC. Accumulating evidence indicates significant alterations in the immune response occur during cirrhosis that may account for the development and progression of the condition.

ARC patients have been reported to be in an immunodeficient state, attributed to various non-specific immune abnormalities including defective neutrophil and macrophage function (Rajkovic and Williams 1986, Gomez et al. 1994), leading to an increased susceptibility to infection. At the same time, several studies have demonstrated elevated levels of pro-inflammatory cytokines in patients with liver cirrhosis, which have been correlated with microbial parameters and clinical complications (Byl et al. 1993, Le Moine et al. 1994, Genesca et al. 1999, Albillos et al. 2004).

The gut microbiota is a metabolically active population of organisms complementing digestion and absorption of nutrients in order to preserve their mutualistic existence in the body. However, it has been reported that the metabolism of ethanol to its toxic metabolite, acetaldehyde, results in the derangement of a previously impenetrable endothelial barrier. This disruption subsequently results in the translocation of gut-derived endotoxins to the portal circulation (Hines and Wheeler 2004, Rao 2009). Moreover, it has been reported that inflammation caused by the metabolites of ethanol, reactive oxygen species (ROS), aldehyde modified proteins or lipopolysaccharide (LPS) increase the levels of cytokines/chemokines resulting in a deleterious positive feedback loop that propagates liver inflammation, infiltration of inflammatory cells (Day and James 1998, Ma et al. 2008) and fibrosis. In support of this, aldehyde modified proteins (Rolla et al. 2000, Vidali et al. 2008) and endotoxin

(LPS) have been detected in the serum and/or livers of patients with liver disease secondary to alcohol. Furthermore, these substances have been shown to increase the release of TNF-a, interleukin-1β, and prostaglandin by kupffer cells, sinusoidal endothelial cells and stellate cells. In addition, increased secretion of inflammatory cytokines by DCs (Laso et al. 2007, Almeida et al. 2013) together with the activation of cytotoxic T and NK cells have also been reported (Laso et al. 1997, Jerrells 2002, Cook et al. 2004, Zheng and Rudensky 2007) further emphasizing the pro-inflammatory state of play.

Of note, evidence from rat studies also indicates that ethanol-induced 'gut leakiness' and endotoxaemia precedes the liver inflammation, with subsequent cytokine dysregulation (Keshavarzian et al. 2009). In view of studies in support of the role of Tregs in immunoregulation (Zheng and Rudensky 2007, Miyara and Sakaguchi 2011) it is reasonable to hypothesise that defects in Treg numbers may also be involved in the perpetuation of such an immune response seen in ARC.

In this chapter, I have further shown a correlation with disease severity and the impaired immunoregulatory function of Tregs **(Figure 3.4A).** Therefore, it is feasible to also postulate that the defect in the Treg suppressor function can allow for the unopposed activation of various inflammatory cells, particularly: Kupffer cells, DCs and monocytes, in the setting of ARC (Houot et al. 2006). In agreement, studies have already shown the role of Tregs in inhibiting the activation, proliferation and effector functions of several populations of immune cells, including $CD8^+$ cytotoxic T cells, NK cells and NK T cells (McNally et al. 2011).

As such the reduced numbers and function of the Tregs seen in ARC can contribute to the increased activation of such lymphoid cell populations, further fuelling the inflammatory environment.

Interestingly, however, a positive feedback loop may be at play, whereby the increased proinflammatory environment can in turn alter the Treg function by resulting in the plasticity of these cells in patients with ARC. In support of the plasticity of Tregs *in vivo*, studies have shown that in patients with alcohol related liver disease there is a predominance of IL-6 cytokine, a cytokine known to inhibit the differentiation of Tregs and induce a Th17 T cell response (La Cava 2008).

In line with this, it has been shown that ALD is associated with an activation of the IL-17 pathway (Lemmers et al. 2009). In agreement with these reports, on further characterisation of isolated Tregs from ARC patients it was further shown that the culture of these cells in the presence of Th17 skewing conditions led to an increase in the percentage of IL-17^{+}FOXP3^{+} cells **(Figure 3.7A).** Furthermore, on phenotypic characterisation of Tregs from ARC patients, a higher percentage of CD161^{+} Treg as compared to HCs was noted. This marker on Tregs has previously been reported to denote Tregs with the propensity to produce IL-17, as well as being described as a marker for precursors of IL-17 producing T cells (Cosmi et al. 2008, Maggi et al. 2010, Afzali et al. 2013, Pesenacker et al. 2013). These results are suggestive of the susceptibility of these cells to differentiate into Th17 cells under the inflammatory conditions described in patients with ARC.

In view of the importance of Tregs in this setting and the potential use of these cells for adoptive cell therapy, the mechanism for the Treg dysfunction in patients with

ARC, was next investigated. The motivation being that further understanding of the process/pathway involved may guide future treatment modalities.

The chronic state of inflammation teamed with a heightened state of oxidative stress, crucial in the pathogenesis of cirrhosis, directed the work as to determine whether there was a pathway that could orchestrate the two processes and explain the Treg dysfunction.

Accumulating evidence supporting the pivotal importance of HO-1 expression in mediating antioxidant and antiapoptotic effects (Brouard et al. 2002, Lee and Chau 2002) and further work suggesting that human $CD4^{+}CD25^{+}$ cells constitutively express HO-1 (Pae et al. 2003) warranted the assessment of HO-1 expression in ARC patient Tregs. This venture was further supported by the similarities in the anti-inflammatory functions attributed to Tregs and HO-1 enzymatic activity, raising the possibility that HO-1 may also be a key mediator of Treg activities. It was shown that

Tregs from ARC patients expressed HO-1, although at significantly lower levels when compared with HC Tregs. As such, it was postulated that the defects seen in Treg numbers and function in patients with ARC may also be attributed to increased apoptosis *in vivo* secondary to the oxidative stress (Alderman et al. 2002, Witko-Sarsat et al. 2003, Baskol et al. 2006, Fialova et al. 2006)

In agreement, Choi et al. have shown that overexpression of HO-1 in Jurkat T cell lines, makes the cells resistant to Fas-mediated apoptosis and further showed that iron, one of the products of the heme degradation by HO-1, is critical for the survival of these cells. In the same study they further confirmed the importance of HO-1

expression against activation induced cell death in primary human $CD4^+$ T cells (Choi et al. 2004). In this study, I showed that $CD4^+CD25^-$ effector cells from patients expressed HO-1 at normal levels, suggestive that the Tregs are particularly sensitive to apoptosis in the environment in vivo, as compared their $CD4^+CD25^-$ counterparts.

It is well known that pro-inflammatory mediators such as TNF-α, IL-1, LPS and oxidized lipids are potent inducers of HO-1 expression in endothelial cells and macrophages (Camhi et al. 1998, Wagener et al. 2003). In this regard, it is reasonable to postulate that in patients with ARC, in view of the increased LPS in the serum and its associated proinflammatory milieu, the lack of HO-1 induction may be due to defects in the signalling pathways involved in HO-1 gene expression.

Other than the antiapoptotic role of HO-1, of particular interest are publications to suggest that HO-1 is important in FOXP3 mediated immune suppression with reports showing that FOXP3 can induce HO-1 expression in a human T cell line (Choi et al. 2005). Several studies have already tested this hypothesis through the use of various *in vitro* assays whereby application of exogenous carbon monoxide (CO), a byproduct of heme metabolism by HO, to murine (Song et al. 2004) and human $CD4^+$ T cells *in vitro* has been shown to mimic Treg suppressive effects. Moreover, the suppressor activity of human Treg appeared enhanced or reduced when HO-1 expression and activity were induced or repressed, respectively (Choi et al. 2005). However, the evidence for the role of HO-1 in Treg mediated suppression in human and animal models are contradictory (Brusko et al. 2005, Zelenay et al. 2007, Biburger et al. 2010).

Here I provided further supporting evidence to the study by Choi et al. for the involvement of HO-1 in Treg mediated immunoregulation. ZnPP, an inhibitor of HO-1 activity, decreased the suppressive function of the Tregs, as assessed by the proliferation of the Teffs.

The data presented here, highlights the important role of Tregs in view of their functional defect, associated with the relative expression of HO-1, and their potential role in the disease progression of ARC. Therefore, to conclude, the defect in HO-1 expression in Tregs may have important implications in aggravating the progressive inflammatory cascade seen in patients with ARC. As such this may present an attractive potential therapeutic target in preventing/slowing the disease progression in this condition.

Chapter 4

EX VIVO EXPANSION OF REGULATORY T CELLS FROM PATIENTS WITH END STAGE LIVER DISEASE; INCREASED SUPPRESSOR FUNCTION AND CLINICAL APPLICATION

4.1. Introduction and Objectives

In view of murine models reporting an abundance of Tregs in tolerated liver allografts and their depletion resulting in the loss of tolerance (Li et al. 2006), it is evident that an increased number of Tregs is needed to tip the balance in favor of regulation. Concurrently in human subjects, it has been shown that stable liver allografts are abundant with such cells, and have been observed to increase after the resolution of a rejection episode (Demirkiran et al. 2007). Conversely, during acute liver transplant rejection, Treg cells were found to be diminished in the peripheral blood and instead localized to the liver (Stenard et al. 2009). As such it is evident that to achieve transplantation tolerance, large number of functional Tregs are required.

Moreover, recent advances permitting the expansion of Tregs *ex vivo* presents an attractive opportunity in modulating immune responses, through their adoptive transfer. In this regard, the effective implementation of Treg therapy in transplantation is reliant on robust Treg manufacturing plans for the isolation and expansion of a functional and stable Treg product from patients who will be receiving the therapy.

To date, there have not been any studies detailing the isolation and expansion of clinical grade autologous Tregs from liver transplant recipients. However, the GMP manufacture of patient-derived Tregs is of paramount importance in view of the start of trials such as ThRIL. It may well be that an impaired expansion profile of the isolated cells from patients awaiting liver transplantation may become apparent and

by its very nature the prospects of conducting a trial such as ThRIL may become an unrealistic endeavor.

As such, here a protocol to isolate and expand Tregs at GMP standards was employed, with evaluation of the phenotype and function of these cells post expansion as a prelude for the ThRIL trial (NCT02166177). In addition, based on the data presented in chapter 3, it was also pertinent to ensure that not only was it possible to expand Tregs, reaching numbers needed for their clinical application, but that a functional and stable population of these cells was being expanded.

This chapter describes the manufacture of clinical grade patient-derived Tregs from patients with ARC, for the programme of cell therapy set out in the ThRIL trial.

In this regard, the hypothesis under investigation was: a rapamycin based GMP expansion protocol will permit the successful isolation and expansion of a functional and stable Treg cell product, reaching numbers needed for their clinical translation.

The specific objectives of this chapter were to:

1. Isolate Tregs, using a GMP compatible protocol reliant on CD8 depletion and CD25 enrichments, from patients with ARC.

2. Polyclonally expand the patient-derived Tregs over 36 days in the presence of rapamycin, using a GMP compatible expansion protocol.

3. Assess the phenotype, function and stability of the *ex vivo* expanded Tregs to ensure the enrichment of a pure and functional population of cells for cell therapy application.

4.2. EFFICIENT ISOLATION AND EXPANSION OF A PURE REGULATORY T CELL POPULATION FROM PATIENTS WITH ALCOHOL RELATED CIRRHOSIS

In preparation for the ThRIL trial, a GMP compatible protocol was firstly used to isolate clinical grade autologous Tregs from patient with ARC. As such, 150ml of peripheral blood was obtained from 9 patients with ARC and 9 age and sex matched HCs. Tregs were obtained following $CD8^+$ cell depletion and enrichment for $CD25^+$ cells, using GMP grade reagents and magnetic beads (Miltenyi Biotec) (Peters et al. 2008, Afzali et al. 2013). Using this protocol, similar numbers of cells were isolated from ARC patients and HCs respectively, $12.6x10^6 \pm 2.25$ compared with $14.4x10^6 \pm 2.57$, p=0.702 **(Figure 4.1).**

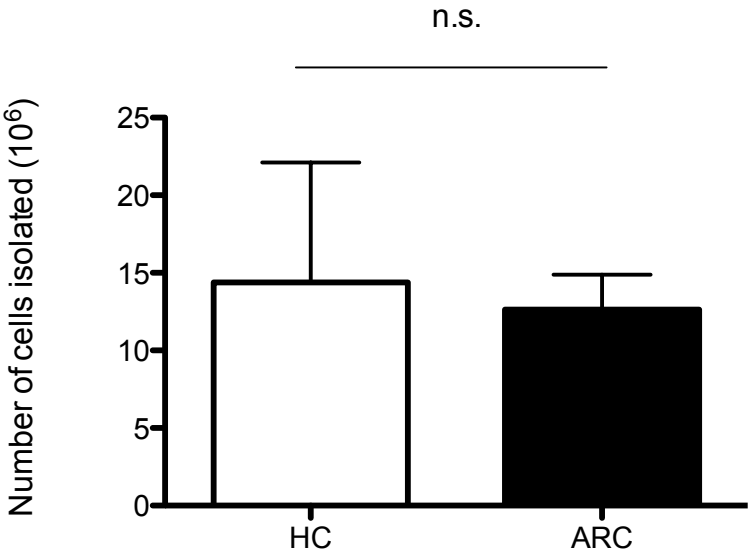


FIGURE 4.1. GMP TREG ISOLATION

A. Numbers of cells isolated from 150ml of blood by $CD8^+$ cell depletion and $CD25^+$ cell enrichment compared between 9 ARC patients and 9 HCs. *** p=<0.001. *Abbreviation, n.s-not significant.* Error bars represent SEM.

One of the difficulties governing the feasibility of adoptive Treg cell therapy is their relative paucity in the peripheral blood, further exaggerated in patients with ARC who have an even lower frequency of Tregs as compared to HCs (data shown in chapter 3). In order to increase the number of Tregs for therapeutic use, *ex vivo* expansion prior to their clinical application is required. In this regard, the large scale *ex vivo* expansion of human Tregs by stimulation with anti-CD3/CD28 monoclonal antibody-coated beads, high dose IL-2 and rapamycin has already been demonstrated successfully in my laboratory (Afzali et al. 2013, Scotta et al. 2013).

Assessment of the purity of the cultures, evaluated by flow cytometric analysis of the percentages of cells expressing $CD8^+$, $CD4^+$ and $CD25^+$ molecules, demonstrated on average a purity of 77.7% ± 10.3 $CD4^+CD25^+$ and 2.50 % ± 1.71 $CD8^+$ cells at S1 for the 9 Treg lines. The data further supported the expansion of a pure population of cells with an increase in percentage of $CD4^+CD25^+$ T cells in culture and diminished levels of $CD8^+$ T cells by final harvest. ($CD4^+CD25^+$ at final harvest, **Untreated;** 87.5% ± 4.12, p=0.088 and **Rapamycin;** 91.3% ± 2.33, p=0.004; $CD8^+$ at final harvest, **Untreated;** 0.292% ± 0.172, p=0.0019, **Rapamycin:** 0.153% ± 0.073 p=0.008 **(Figure 4.2A, 4.2B).**

In addition, since the cell sorter does not currently have GMP certification here in the UK, the cells could not be isolated based on $CD25^{Hi}$ expression. Despite this, the expansion protocol used resulted in the selective expansion of $CD25^{Hi}$ and, as evident from the dot plot **(Figure 4.2B)**, the expression of $CD25^{Hi}$ was highest following exposure of Tregs to rapamycin, conforming with previous reports (Golovina et al. 2011).

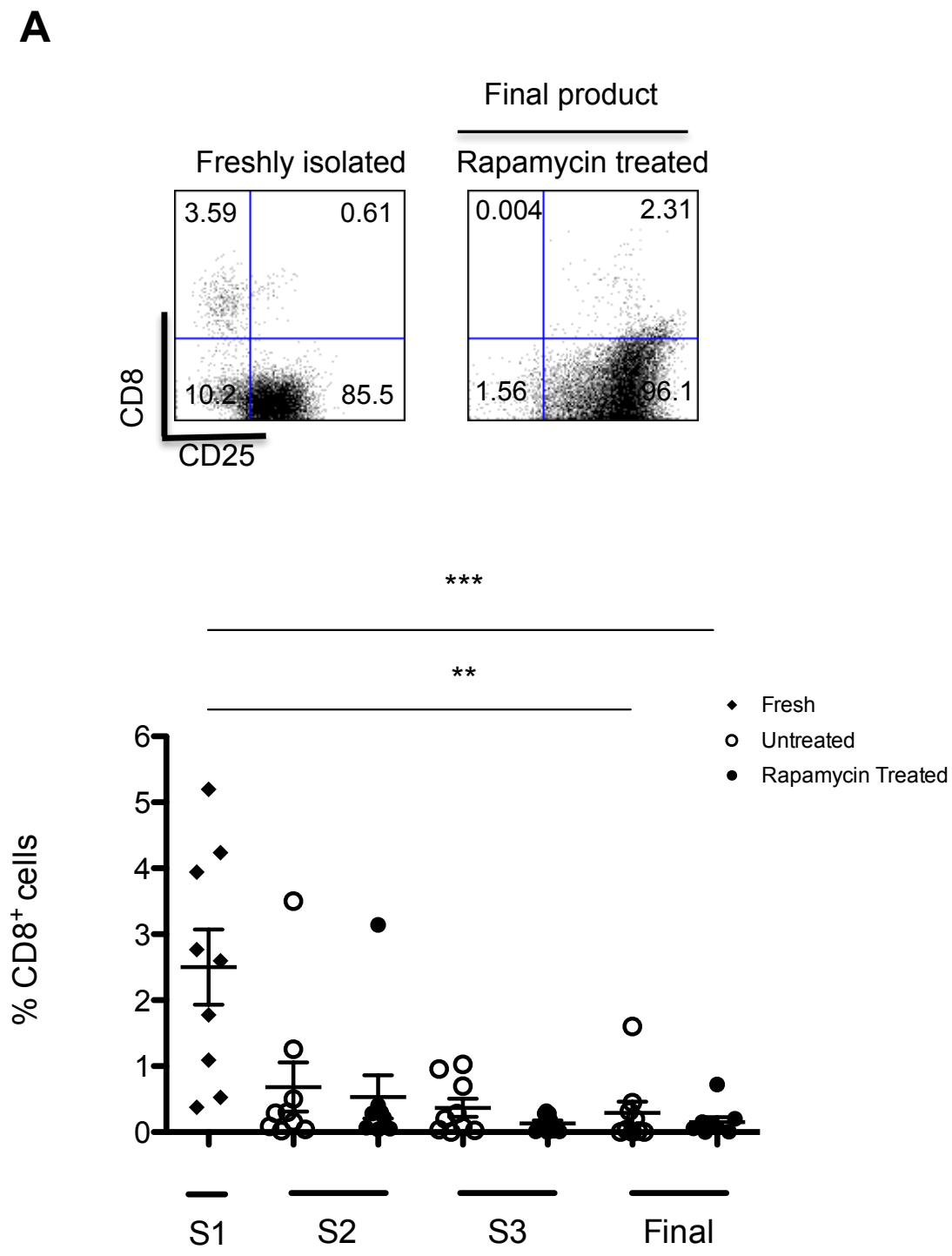


FIGURE 4.2. A GMP COMPATIBLE TREG ISOLATION TECHNIQUE, RELIANT ON A TWO STEP PROCESS

A. $CD8^+$ cell depletion. n=9 ARC patients. S1-day 0, S2- day 12, S3- day 24, final- day 36. **p=<0.05, ***p=<0.001. *Abbreviation, S-stimulation,* n.s- not significant. Error bars represent SEM.

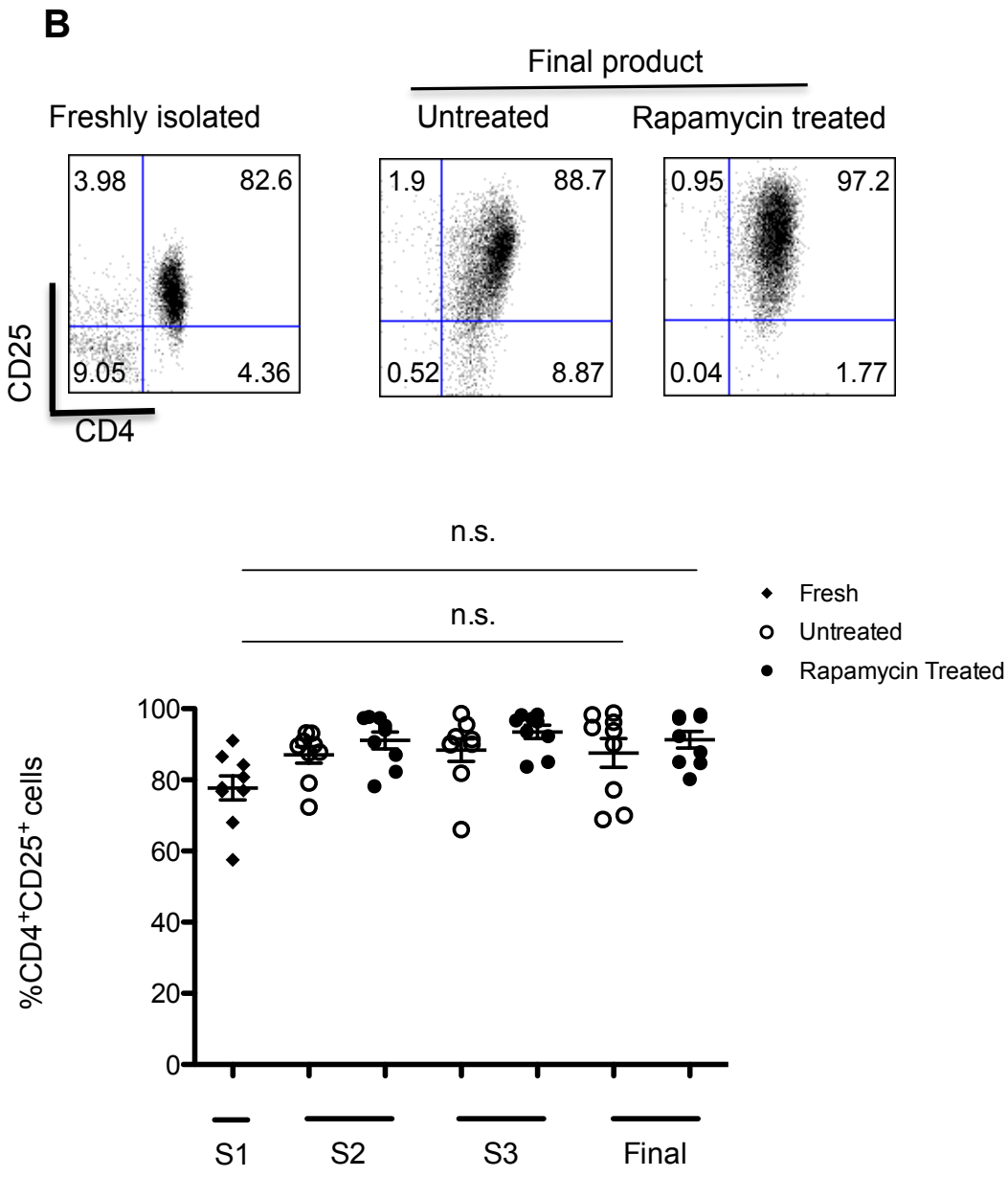


FIGURE 4.2. A GMP COMPATIBLE TREG ISOLATION TECHNIQUE, RELIANT ON A TWO STEP PROCESS

B. CD25$^+$ cell enrichment. In each case dots plots are representative of 9 ARC patients. Graphs denote the purity of the culture throughout the expansion period in both rapamycin and untreated cultures. S1-day 0, S2- day 12, S3- day 24, final -day 36. n= 9. *Abbreviation, S-stimulation, n.s.- not significant*. **p=<0.05, ***p=<0.001. Error bars represent SEM.

4.3. REGULATORY T CELLS FROM PATIENTS WIH ALCOHOL RELATED CIRRHOSIS CAN BE EXPANDED TO CLINICALLY SUITABLE NUMBERS

Data from animal models have clearly demonstrated that for Tregs to suppress immune responses a high Treg to T effector ratio is required (Hoffmann et al. 2002, Taylor et al. 2002).

Having established that a pure population of Tregs can be isolated and expanded from ARC patients, I next sought to determine whether they can be expanded *in vitro*, under GMP compatible conditions, to numbers required for the maximum dose of Treg injection planned for the dose escalation ThRIL trial ($4.5x10^6$/Kg).

Freshly isolated Tregs from the 9 ARC patients and 9 HCs were expanded *in vitro* in the presence and absence of rapamycin. Tregs from both groups expanded rapidly with comparable expansion profiles between ARC patient Tregs and HCs during the 36 days of culture (Fold expansion **Untreated**; ARC 2080 ± 428, HC 1670 ± 359 p=0.469; **Rapamycin;** ARC 1430 ± 239 and HC 1060 ± 139 p= 0.207) **(Figure 4.2C)**. In addition, it has previously been reported that the addition of rapamycin to cultures can delay the kinetics of proliferation (Battaglia et al. 2005, Hippen et al. 2011), conceivably necessitating a prolonged period of culture to achieve the doses suitable for clinical application. Here, I provide data that at final harvest, despite a lower fold expansion of Tregs in the presence of rapamycin this difference was not significant when compared to the untreated cultures in both cohorts (**ARC**; Rapamycin 1427 ± 239 vs Untreated 2082 ± 428, p=0.199; **HCs;** Rapamycin: 1062 ±

139 vs Untreated: 1668 ± 359, p= 0.135). Of note, the average expansion of the 9 different Treg lines in the presence of rapamycin was 11.2 x 10^9 cells ± 2.59 **(Figure 4.2D),** demonstrating the feasibility of reaching numbers needed for the high dose of Tregs planned to be tested in the ThRIL trial.

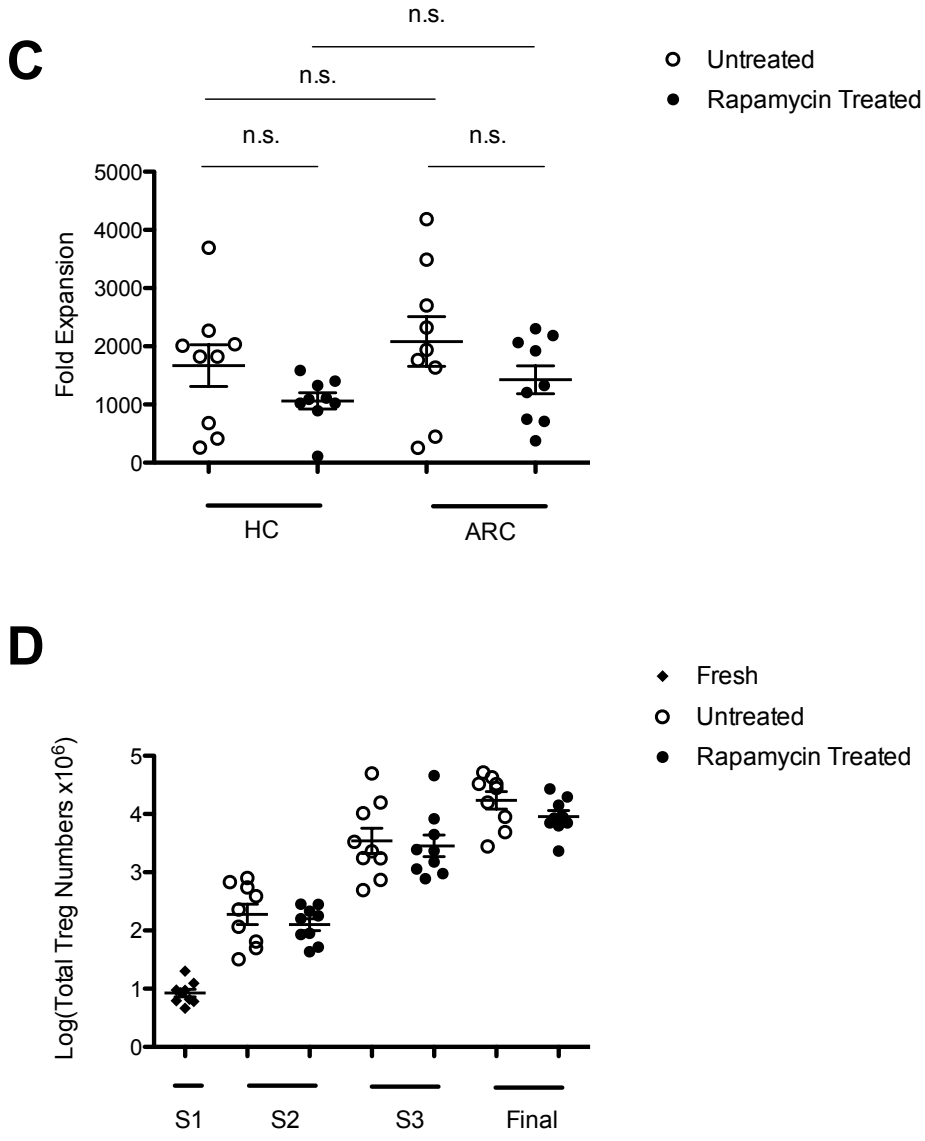


FIGURE 4.2 GMP COMPLIANT POLYCLONAL TREG EXPANSION

C. Comparison of Treg expansion profiles between HCs and ARC patients following 36 days of culture. Tregs from 9 ARC patients and 9 HCs were expanded over 36 days in the presence and absence of rapamycin. Fold expansion was calculated from Treg numbers at each stimulation. **D. Treg numbers over the 36 day expansion period.** Graph denoting the number of ARC patient Tregs at each stimulation, based on fold expansion and assuming that all cells were expanded at each stimulation. S1-day 0, S2- day 12, S3- day 24, final -day 36. *Abbreviations, S- stimulation, n.s- not significant*. **p=<0.05, ***p=<0.001. Error bars represent SEM.

4.4. Regulatory T cells expanded in the presence of rapamycin maintained high levels of FOXP3 and CTLA4 with a sustained expression of CD62L and CXCR3.

To verify the isolation and enrichment of the Tregs, freshly isolated and expanded Tregs were analysed for the expression of the transcription factor, FOXP3, of importance in Treg development and function (Hori and Sakaguchi 2004). The percentage of $CD4^{+}CD25^{+}FOXP3^{+}$ cells was 92.1% ± 2.99 at baseline, and contrary to published data reporting a loss of FOXP3 expression with prolonged periods of culture (Hoffmann et al. 2009), the data showed that the expression of FOXP3 was maintained in culture in the presence (94.6% ±2.44) and absence (92.9% ±2.15) of rapamycin **(Figure 4.3A).**

In addition, and in support of the supplementation of cultures with rapamycin, with a more stringent gating strategy applied **(Figure 4.3B)** it was shown that the percentage of $CD4^{+}CD25^{+}FOXP3^{Hi}$ positive cells was increased in culture in the presence of rapamycin (S1: 24.68% ± 2.95 vs Final harvest: 63.4% ± 4.23, $p< 0.0001$) as compared to the untreated cultures **(Figure 4.3C).** In agreement, the mean fluorescent intensity (MFI) of FOXP3 expression was also highest following exposure to rapamycin as compared untreated cultures and baseline **(Figure 4.3D).**

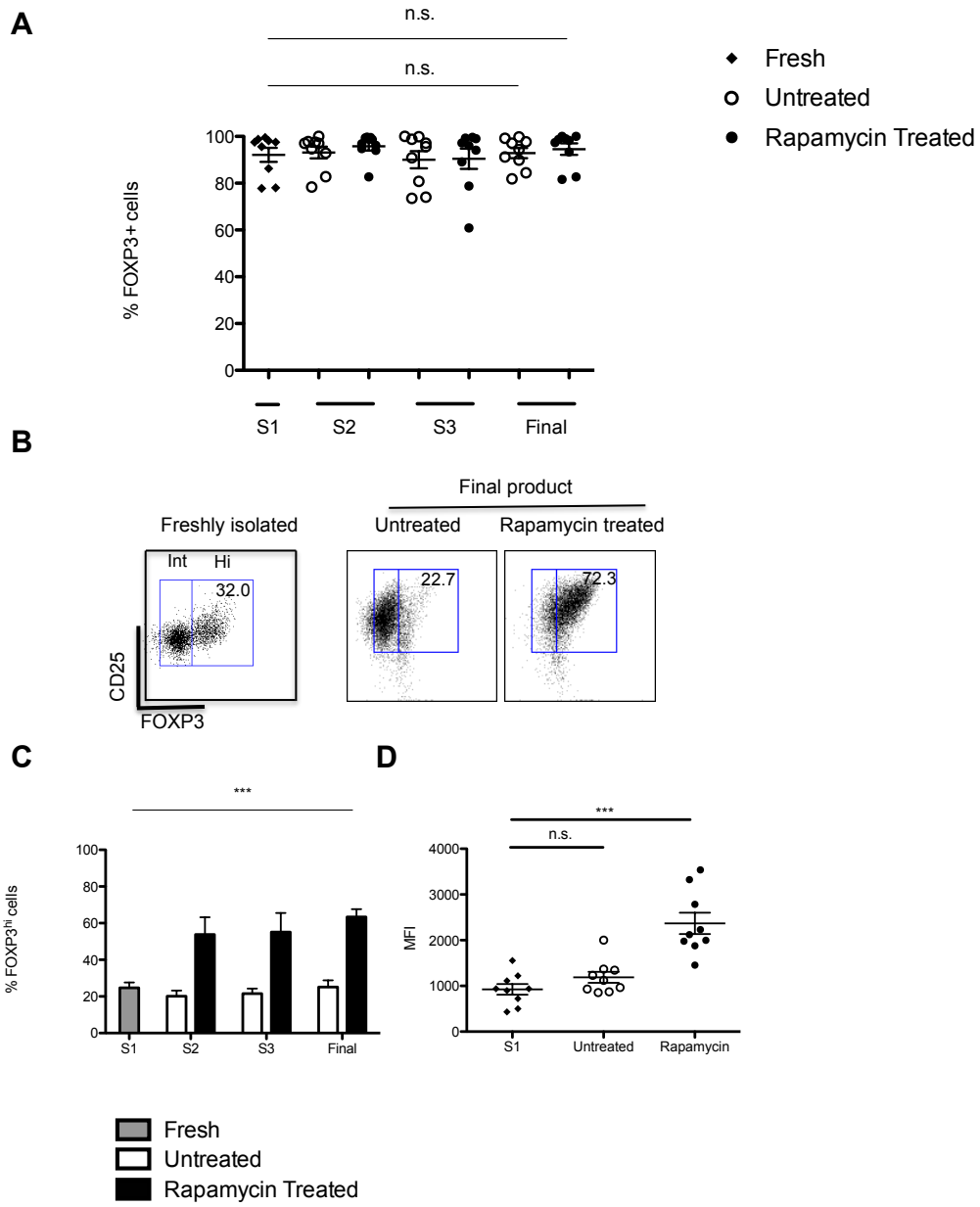


FIGURE 4.3. EXPRESSION OF FOXP3 BY TREGS THROUGHOUT CULTURE

A. Frequency of FOXP3$^+$ Tregs. Graph shows the mean percentage of CD4$^+$CD25$^+$ cells expressing FOXP3$^+$ in both untreated and rapamycin treated cultures of 9 ARC patients over 36 days of culture. **B. Flow cytometric gating strategy used to define two populations of FOXP3$^+$ cells.** A representative plot depicting the stringent gating strategy applied to delineate a population with a high expression of FOXP3 (Hi) and one with intermediate expression of FOXP3 (Int). **C. Frequency of FOXP3^{Hi} Tregs throughout culture.** Graph shows the frequency of FOXP3^{Hi} Tregs, from 9 ARC patients, throughout culture ± rapamycin**. D. Mean fluorescent intensity (MFI) of FOXP3 expression by Tregs.** FOXP3$^+$ MFI within gated CD4$^+$ CD25$^+$ cells in 9 ARC patients. MFI shown at day 0 and at final harvest for Tregs expanded in both the presence and absence of rapamycin. S1-day 0, S2- day 12, S3- day 24, final-day 36. *Abbreviations, S- stimulation, n.s.-not significant*. ***p=<0.001. Error bars represent SEM.

Apart from FOXP3, a constitutive high expression of the CTLA-4 represents another well-documented characteristic of $CD4^+CD25^+$ Tregs that has also been shown to contribute to their suppressive function (Read et al. 2000, Annunziato et al. 2002, Sansom and Walker 2006). Analysis of the cultures at final harvest revealed that rapamycin led to a significant increase in the percentage of Tregs expressing CTLA-4 (S1 15.3% ± 2.51 vs Final harvest 70.6% ± 4.522, p <0.0001) **(Figure 4.4A)** with an increase also evident in the MFI of this marker **(Figure 4.4B).**

An additional consideration regarding Treg therapy is the site of action of Tregs and, consequently, the desired homing properties of the injected cells. In the transplant setting, Treg lymph node homing and their ability to traffic to grafts are both required for their protection against graft rejection (Ochando et al. 2005). In this regard, it was shown that cultures of Tregs in the presence of rapamycin maintained the expression of the lymphoid homing receptor, CD62L (S1: 84.5% ± 1.67 and Final: 85.2% ± 1.45, p=0.738), which was not preserved in the untreated cultures, p=0.0076 **(Figure 4.5).**

Furthermore, the percentage of Tregs expressing CXCR3, the chemokine receptor important for the migration and recruitment of Tregs to the liver (Oo et al. 2010) at baseline and after the 36 days expansion was investigated. Our data concluded a significant decrease in the Tregs expressing CXCR3 in the untreated cultures as compared baseline, p= 0.0194. This is in keeping with previous studies showing changes in the expression of the homing receptors with prolonged periods of culture (Chakraborty et al. 2012). However, it was clearly demonstrated that using the rapamycin based expansion protocol the expression of this marker on the Tregs was maintained **(Figure 4.5).**

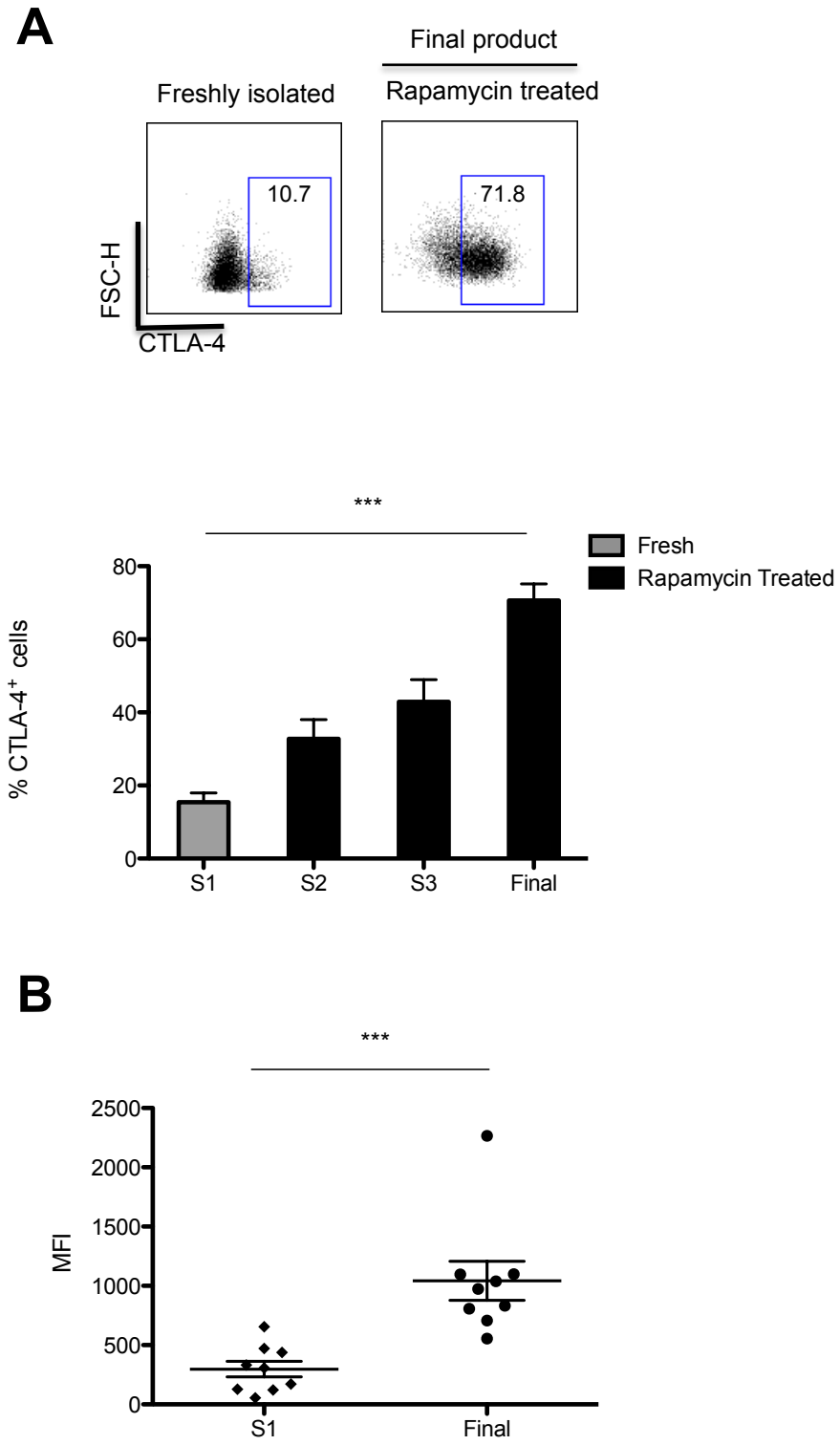


FIGURE 4.4. EXPRESSION OF CTLA-4 THROUGHOUT CULTURE

A. Gating and Frequency of CTLA-4$^+$ Tregs. Dot plot details the frequency of CTLA-4 expression on CD4$^+$CD25$^+$ Tregs from one representative sample of 9 ARC patients. The graph depicts the frequency of CD4$^+$CD25$^+$CTLA-4$^+$ Tregs throughout culture in the presence of rapamycin. **B** MFI of CTLA-4 expression at S1 and day 36 of culture in the presence of rapamycin. ***p=<0.001. S1-day 0, S2- day 12, S3- day 24, final-day 36. Error bars represent SEM.

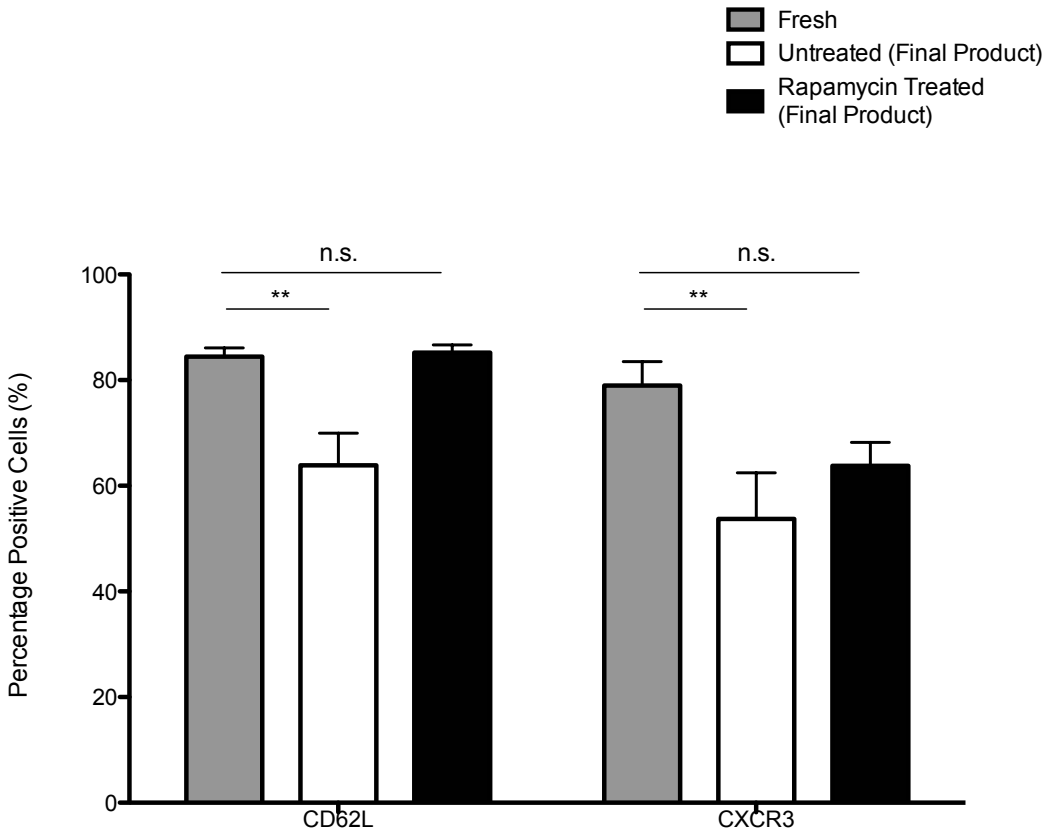


FIGURE 4.5. TREG HOMING; FREQUENCY OF CD62L^{+}AND CXCR3 BY TREGS.

Graph shows relative expression of CD62L and CXCR3 on CD4^{+}CD25^{+} Tregs, from 9 ARC patients, at day 0 and at final harvest (day 36) in both the presence and absence of rapamycin. *Abbreviations, n.s.-not significant.* **p=<0.01. Error bars represent SEM.

4.5. RAPAMYCIN EFFECTIVELY PREVENTS INTERLEUKIN-17 PRODUCTION BY THE EXPANDED REGULATORY T CELLS FROM PATIENTS WIH ALCOHOL RELATED CIRRHOSIS

One of the major concerns in Treg immunotherapy is the plasticity of the Tregs and their conversion to cells producing inflammatory cytokines. In this regard, several studies have shown that Tregs can convert to a Th17 phenotype, producing IL-17 when exposed to a pro-inflammatory milieu (Hori 2010, Li and Boussiotis 2013). As such, freshly isolated and expanded Tregs were cultured for 5 days in the presence of Th17 skewing conditions, with the percentage of IL-17^{+} cells analysed by FACS **(Figure 6A**) and IL-17 production measured by ELISA **(Figure 6B)** of the culture's supernatant. Our data clearly confirmed that over the 36 day period culture and in the presence of rapamycin, there was a decrease in the percentage of positive cells for IL-17 (**Mix 1**: S1: 5.71% ± 0.98 and rapamycin at final harvest: 2.84% ± 0.809, p=0.0384; **Mix 2**: S1: 4.93% ± 1.12 and rapamycin at final harvest: 2.31% ± 0.436, p=0.0446) and diminished IL-17 production **(Mix 1:** S1: 1965pg/ml ± 318 and rapamycin at final harvest: 124pg/ml ± 34.5, p<0.0001; **Mix 2**: S1: 1322pg/ml ± 347 and rapamycin at final harvest: 84.3pg/ml ± 35.3, p=0.0027) **(Figure 6A, 6B).** Moreover, assessment of the percentage of IFNγ^{+} cells also clearly demonstrated that rapamycin resulted in a reduction in the frequency of FOXP3^{+}IFNγ^{+} cells by final harvest (**Mix 1** S1: 6.42% ± 0.915 and rapamycin at final harvest: 2.54% ± 0.560, p=0.0023; **Mix 2**: S1: 4.61% ± 0.698 and rapamycin at final harvest: 2.82% ± 0.688, p=0.084) (**Figure 6C**).

To better characterise the Tregs and ensure that the expansion protocol favored the stability of these cells in culture, the expression of CD161 on the Tregs was also investigated. My laboratory and others, have previously shown that Tregs expressing CD161 are responsible for IL-17 production (Cosmi et al. 2008, Afzali et al. 2013, Pesenacker et al. 2013), as well as being described as a marker for precursors of IL-17 producing T cells (Maggi et al. 2010).

I show that during the 36-day expansion there was a decrease in the percentage of $CD161^+$ Tregs **(**S1: 19.6% ± 2.42 and rapamycin at final harvest: 1.48% ± 0.26, p=0.0001) and a decrease in the percentage of Tregs co-expressing CD161 and CCR6 (S1: 12.5% ± 1.75 and rapamycin at final harvest: 0.500 ± 0.107, p<0.0001) in rapamycin treated cultures as compared to baseline **(Figure 4.6D, 4.6E).**

Taken together these findings suggest that expansion of Tregs with rapamycin decreased their capacity to produce IL-17 under inflammatory conditions.

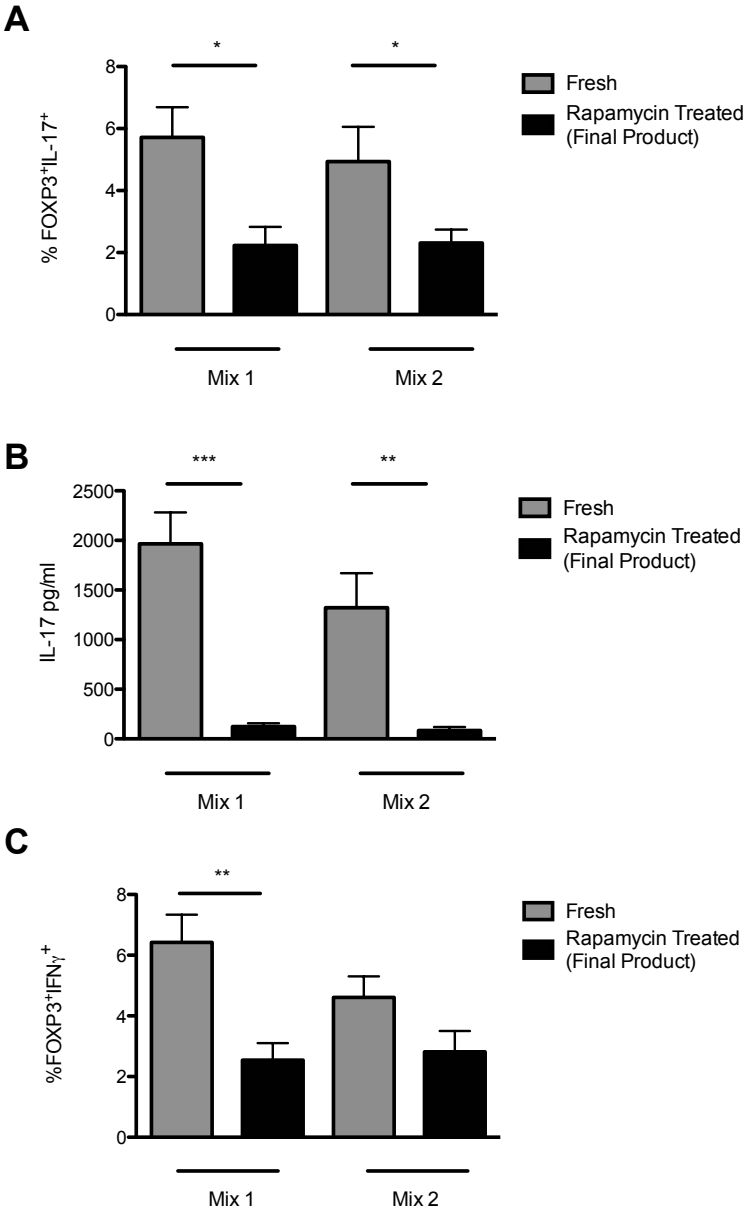
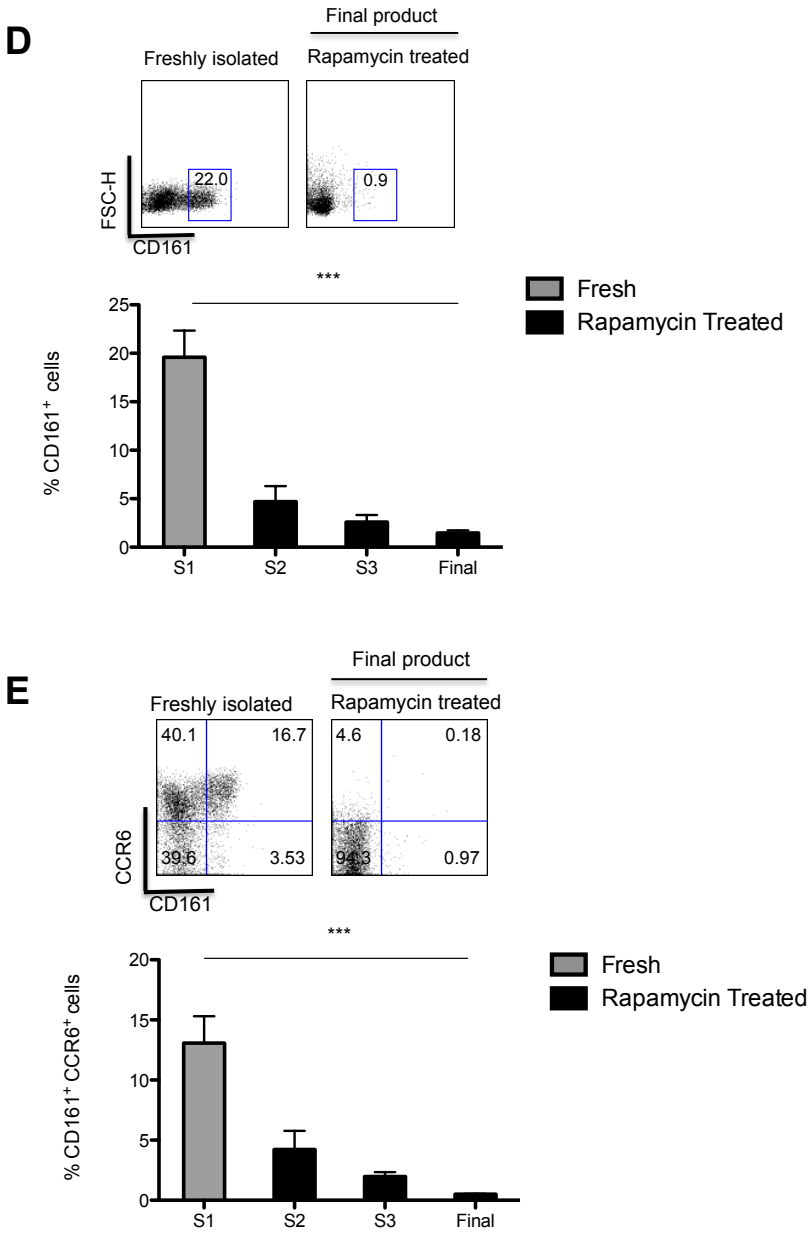


FIGURE 4.6. CUMULATIVE DATA FROM 9 INDIVIDUAL EXPERIMENTS DETAILING THE INTRACELLULAR EXPRESSION OF IL-17 AND IFN-Y AND PRODUCTION OF IL-17.

A. Graph showing the frequency of IL-17$^+$ in CD4$^+$CD25$^+$FOXP3$^+$ Tregs upon isolation and post culture in the presence of rapamycin (day 36) when exposed to two separate mixes of pro-inflammatory cytokines (**Mix 1**: IL-2, IL1β, IL-6 and TGF-β and **Mix 2:** IL-2, IL-21, IL-23 and TGF-β) **B.** Production of IL-17 (pg/ml) from CD4$^+$CD25$^+$FOXP3$^+$ Tregs, as assessed by ELISA. **C.** Graph showing the frequency of IFN-γ$^+$ in CD4$^+$CD25$^+$FOXP3$^+$ Tregs upon isolation and post *ex vivo* expansion in the presence of rapamycin (day 36) when exposed to the two separate mixes of pro-inflammatory cytokines (as above). n.s.-not significant. *p=<0.05,**p=<0.01. n= 9 ARC patients. Error bars represent SEM**.**



D. Gating strategy and Frequency of CD161$^+$ Tregs throughout culture. Dot plot depicts expression of CD161 on CD4$^+$CD25$^+$ Tregs from a representative sample of 9 ARC patients. The graph shows the dynamics of CD161 expressing Tregs throughout culture. **E. Gating strategy and Frequency of CCR6$^+$CD161$^+$ Tregs throughout culture** Dot plot of CD161$^+$CCR6$^+$ co-expression on CD4$^+$CD25$^+$ Tregs from a representative sample of 9 ARC patients and graph of percentage CD161$^+$CCR6$^+$ co-expression throughout culture. ***p=<0.001. S1-day 0, S2- day 12, S3- day 24, final-day 36. Error bars represent SEM.

4.7. *EX VIVO* EXPANDED REGULATORY T CELLS FROM PATIENTS WIH ALCOHOL RELATED CIRRHOSIS HAVE INCREASED SUPPRESSIVE ABILITY

After confirming that the cells maintained their stability during culture it was also of importance to assess the functional properties of the expanded patient-derived Tregs. CFSE dilution assay was performed to evaluate the ability of freshly isolated and *ex vivo* expanded Tregs from patients to suppress the proliferation of T effectors **(Figure 4.7).**

In line with previous reports (Scotta et al. 2013), the data clearly demonstrated that at the end of expansion, the suppressive function elicited by ARC Tregs in the presence of rapamycin was significantly higher as compared to freshly isolated Tregs at different Treg:Teffector ratios (**1:1 ratio** - expanded rapamycin Tregs 84.8% ± 3.96 vs. Tregs at S1 25.4% ± 6.03, p=0.0001 suppression, **1:10 ratio** – expanded rapamycin Tregs: 68.3% ± 6.85 vs. Tregs at S1: 16.2% ± 4.68, p=0.0001). In the untreated cultures, despite an increase in Treg suppressive function at S2, this increase did not reach similar levels as those obtained from Tregs expanded in the presence of rapamycin (Treg:Teff- **1:1** ratio – **Untreated:** 60.5% ± 7.26 vs **Rapamycin:** 88.2% ± 2.76, p=0.0026; and **1:10** ratio - **Untreated:** 42.0% ± 8.99 vs **Rapamycin:** 79.1% ± 3.90, p= 0.0016). Moreover, the increase in Treg suppressive function was not preserved by final harvest in the untreated cultures as compared to the cultures expanded in the presence of rapamycin (Treg:Teff 1:1 ratio - **Untreated:** 25.2% ± 4.58 vs **Rapamycin:** 84.8% ± 3.96, p=0.0001; 1:10 ratio - **Untreated** 7.32% ± 2.32 vs **Rapamycin** 68.3 ± 6.85, p=0.0001) **(Figure 4.7A).** These results supported the use of the rapamycin based GMP protocol in the expansion of autologous Tregs from

ARC patients for cell therapy application in view of the increased Treg suppressive function, which was maintained during expansion.

4.8. Regulatory T cell expansion in the presence of rapamycin results in an increased expression of Heme Oxygenase-1 by patient Regulatory T cells.

In view of the role of HO-1, in Treg mediated suppression, described in chapter 3, and data presented suggesting that Tregs from ARC patients have lower expression of HO-1, levels of this enzyme were measured in rapamycin expanded patient derived Tregs by final harvest. The rationale being that if rapamycin treatment results in the increase of the suppressor function of Tregs from ARC patients, in parallel, expression of HO-1 in Tregs from patients will also increase. It was clearly demonstrated that by final harvest, patient Tregs expressed higher levels of HO-1, 6.19% ± 1.05 as compared to the level of HO-1 obtained from freshly isolated patient Tregs, 1.76% ± 0.404, p= 0.0171. **(Figure 4.7B).**

The level of HO-1 observed with rapamycin cultured Tregs from ARC patients was very similar to HO-1 levels in Tregs from HCs, p= 0.3609 **(Figure 4.7C)**. Moreover, and of importance, our preliminary data to date has also indicated that levels of HO-1 from HC Tregs do not increase post 36 days expansion in the presence of rapamycin (data not shown).

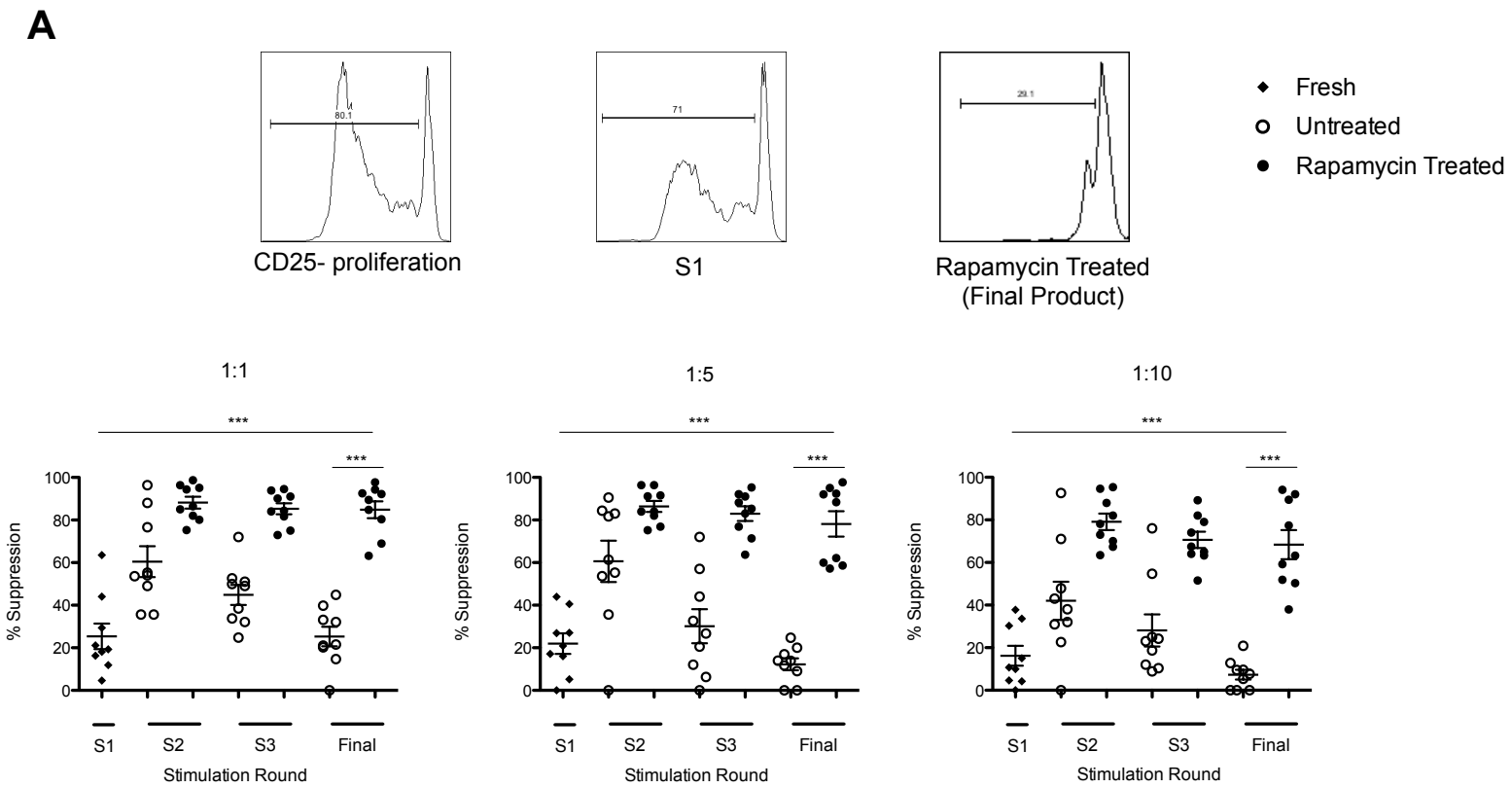


FIGURE 4.7. ASSESSEMENT OF $CD4^+CD25^+$ TREG SUPPRESSOR FUNCTION AND CONCURRENT EXPRESSION LEVELS OF HO-1.

A. Representative histogram and graph from 9 ARC patients upon assessment of Treg suppressor function. The suppressive function of Tregs cultured ± rapamycin was assesed by CFSE dilution assay at day 0 and throughout the 36 day culture period.

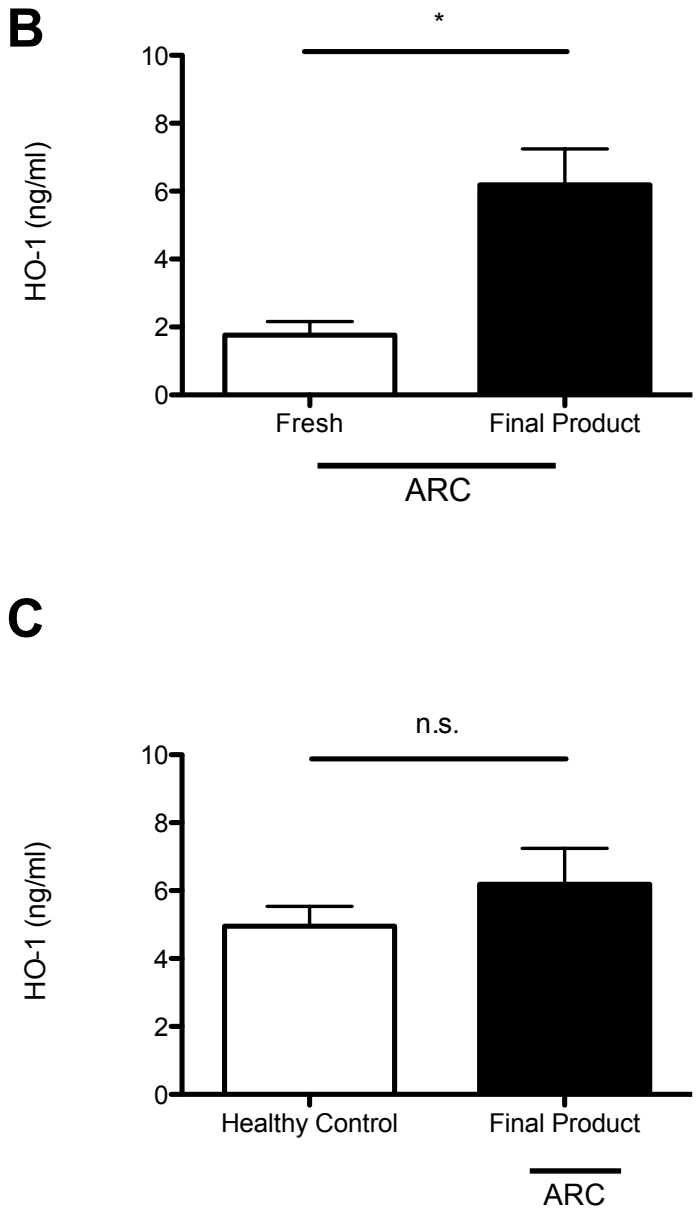


FIGURE 4.7. ASSESSEMENT OF $CD4^+CD25^+$ TREG SUPPRESSOR FUNCTION AND CONCURRENT EXPRESSION LEVELS OF HO-1.

B. Graph depicting the expression levels of HO-1 expression by $CD4^+CD25^+$ Tregs from 3 ARC patients following 36 days of culture in the presence of rapamycin as compared to freshly isolated Tregs from patients. **C.** Graph comparing the mean HO-1 expression of rapamycin expanded Tregs, from 3 ARC patients at final harvest, with the mean expression of HO-1 by HC Tregs. n.s.- not significant. *p=<0.05, ***p=<0.001. S1-day 0, S2- day 12, S3-day 24, final-day 36. Error bars represent SEM.

4.9. DISCUSSION

One of the major drawbacks following liver transplantation is the requirement for lifelong treatment with immunosuppressant exposing their associated side effects. This contributes to a disappointing 60% patient survival rate at 10 years after transplantation. A potential solution to this problem is to reprogram the transplant recipient's immune system so as to maintain normal graft function in the absence of these toxic drugs. In this regard, the use of cell-based therapies, harnessing the natural immunoregulatory properties of the immune system, is an emerging area of science/medicine and Tregs have been recognized as ideal candidates in this endeavor. Animal models and basic science research have already shown the importance of Tregs in the induction of tolerance and prevention of rejection in transplant models (Sakaguchi 2005). In addition, Treg therapy in the context of liver transplantation is supported by studies reporting an increase in the number of $FOXP3^+$ cells in the liver biopsies of operationally tolerant liver transplant recipients (Li et al. 2004, Martinez-Llordella et al. 2007) and low levels of circulating Tregs documented in episodes of acute rejection (Demirkiran et al. 2006). As such, to increase the number of these cells *in vivo* is an attractive concept, tipping the balance in favor of regulation.

ThRIL is the leading clinical trial of autologous Treg immunotherapy in liver transplantation worldwide. The success of such a trial relies on a careful clinical trial design, incorporating a detailed Treg manufacturing plan as well as a 'Treg supportive' clinical protocol that favours the function and survival of these cells *in vivo.*

Here, I outline the first account of the manufacture of Tregs from liver transplant recipients, highlighting the successful isolation and *ex vivo* expansion of these cells at GMP standards. The data presented provides evidence supporting the feasibility of autologous Treg cell-based therapy in liver transplantation.

By adherence to the GMP-compatible protocol devised in my laboratory (Afzali et al. 2013, Scotta et al. 2013) I was able to ensure the selection of a functionally pure population of Tregs ($CD4^+CD25^+$ at S1: 77.7% ± 10.3), using a two-step magnetic activated cell sorting (MACS) protocol. Others have attained a higher degree of Treg purity; using Treg isolation techniques based on the combined expression of $CD4^+$, $CD25^+$, and $CD45RA^+$ molecules, hence eliminating antigen experienced/memory T cells (Hoffmann et al. 2006), or $CD4^+$, $CD25^+$ and low expression of CD127 molecules (Liu et al. 2006). Although cell isolation based on the combination of these markers is highly effective, the lack of GMP cell sorter facilities in the UK makes the isolation of Tregs based on the combination of the three markers unfeasible. In addition, Ukena et al. compared different Treg isolation strategies with the aim of defining the most favorable Treg target population for cellular therapy. They concluded that whilst $CD4^+CD25^+CD127^{lo}$ Tregs are promising for fresh cell infusions, $CD4^+CD25^+$ Tregs qualify as the best candidate for *in vitro* expansion (Ukena et al. 2011). Moreover, Marek et al. showed that during the expansion process Tregs were "transforming" into effector/memory like cells and proposed that regardless of the phenotypic markers used for Treg isolation, the only variable to

maintain Treg phenotype and function is to limit the duration of expansion to 2 weeks (Marek et al. 2011).

In support of this study, others have also shown that the large-scale manufacture of Tregs remains challenging, reporting that even when starting with a highly pure population of Tregs, repeated stimulation results in the loss of FOXP3 expression (Hoffmann et al. 2009). In the clinical trial conducted by Trzonkowski et al. a decrease in the percentage $FOXP3^{+}$ cells after successive weekly stimulation was reported (Trzonkowski et al. 2009). However, the disadvantage of limiting cultures to two rounds of stimulation became evident in the trial of Treg immunotherapy in Type 1 diabetes where the authors noted an insufficient Treg yield in four out of the 10 patients (Marek-Trzonkowska et al. 2012).

Data presented in this chapter details the manufacture of clinical grade Tregs in concordance with the proposed GMP protocol, expanding these cells *in vitro* for 36 days with three rounds of stimulation, in the presence of rapamycin. This resulted in the expansion of Tregs to numbers sufficient for administration of the maximum dose in the program of cell therapy outlined in the ThRIL trial. Despite the three rounds of stimulation, and contrary to the studies outlined above, FOXP3 expression was maintained in culture and the data clearly demonstrated that in the presence of rapamycin there was an increase in the percentage of $FOXP3^{Hi}$ Tregs and in the level of expression of FOXP3 at final harvest **(Figure 4.3C)**. Additionally, the supplementation of cultures with rapamycin has been supported in studies by Battaglia and Strauss showing that rapamycin selectively promotes the expansion of functional human Tregs, whilst depleting human $CD4^{+}CD25^{-}$ T effectors cells (Battaglia et al. 2005, Strauss et al. 2007). Moreover, data from my laboratory and

others have shown that rapamycin has an inhibitory effect on the development of IL-17 producing cells, both *in vitro* and *in vivo* (King et al. 2009, Scotta et al. 2013).

The rapamycin based GMP compatible protocol further proved to be ideal in the expansion of Tregs from liver transplant recipients, promoting an increase and subsequent maintenance of Treg suppressive function throughout the 36-day culture **(Figure 4.7A).**

Ex vivo expansion of Tregs in the presence of rapamycin further led to an increased expression of HO-1 by patient Tregs by final harvest, supporting the increased suppressive function **(Figure 4.7B, 4.7C).** In agreement with this data Visner et al. have suggested that the antiproliferative effects of rapamycin in vascular smooth muscle cells are mediated through the induction of HO-1 (Visner et al. 2003). The study by Kist et al. provides further supporting evidence, showing that culture of rat hepatocytes in the presence of rapamycin led to an increased expression of HO-1, protecting hepatocytes against ROS-induced damage (Kist et al. 2012).

Thus, it may also be suggested that by increasing HO-1 levels by final harvest, it not only results in the increase in Treg suppressive function, but also ensures the resilience of these cells to apoptosis, a desirable characteristic of these cells for adoptive transfer.

In addition, therapeutic strategies using Tregs have to also taken into account the need for appropriate tissue trafficking to enable contact with their target cells. In this respect, in a murine model of T cell mediated liver injury, Lapierre et al. demonstrated the ability of $CXCR3^{+}$ Tregs to target to the liver, in turn potentiating

the effectiveness of Treg adoptive transfer (Lapierre et al. 2013). The data demonstrates the preservation of cells expressing CD62L and CXCR3, important for their migration to lymph nodes and the liver respectively, making the Tregs manufactured with this protocol desirable for application in liver transplantation (**Figure 4.5**).

A major potential barrier to Treg therapy is the possibility that these cells may assume a pro-inflammatory phenotype and cause graft rejection on adoptive transfer. In this study, I confirmed that rapamycin effectively diminished the capacity of Tregs to produce IL-17 (**Figure 4.6A, 4.6B**). In line with this, a decrease in the percentage of cells co-expressing CD161 and CCR6 was further demonstrated. In support of our data Tresoldi et al. showed that only in cultures in the absence of rapamycin $CD4^{+}CCR6^{+}CD161^{+}$ Th17 precursors were present (Tresoldi et al. 2011). Moreover, in view of the plasticity of Tregs, Kopft et al. studied the reciprocal differentiation of Tregs and Th17 cells and reported that rapamycin suppresses the differentiation of pathogenic Th17 cells (Kopf et al. 2007). These studies further confirm the benefits and pertinence of a rapamycin based GMP-compatible protocol when considering translation of autologous Treg based therapy in liver transplantation.

Finally, recently my laboratory have provided data on the large-scale production of patient Tregs in the GMP Clinical Research Facility (CRF) at Guy's Hospital, utilizing the CliniMACS system of Treg isolation, whilst employing the expansion protocol outlined in the present study. In this regard, the successful large scale expansion of Tregs from three liver transplant recipients has been reported, with all final products consistently satisfying the set release criteria **(Appendix 1)** necessary

for their clinical application in the setting of the ThRIL trial (data not shown). Moreover, in view of the fact that the Tregs will be cryopreserved, prior to intravenous infusion, in the ThRIL trial, further data has been provided supporting the stability of the Tregs, retaining the necessary product specifications post the freeze-thaw process. The data generated also has important implications for future clinical trials. The optimal Treg dose level for efficacy in solid organ transplantation is currently unknown. It may well be that multiple Treg injections are required in future Phase II efficacy studies, hence supporting the importance of Treg cryopreservation.

The clinical trial, ThRIL, is currently in the recruitment stage with plans to test the *in vivo* safety and efficacy of the Tregs expanded under the conditions described in this manuscript. In addition, the clinical protocol for this trial is based on a Treg supportive immunosuppressive regimen including the use anti-thymocyte globulin (ATG), to induce lymphopenia with a preferential preservation of Tregs (Lopez et al. 2006) and rapamycin, to promote selective Treg expansion *in vivo* (Strauss et al. 2007). The intention behind this strategy: to create a tolerogenic milieu thus maximizing the potential efficacy of the exogenously administered Tregs through prolongation of their *in vivo* survival. Tailoring the immunosuppressive regimen along with the administration of *ex vivo* expanded Tregs may potentially maintain post liver transplant tolerance, accomplishing the ultimate aim of the ThRIL trial.

Adoptive cell therapy in the setting of liver transplantation is rapidly becoming a reality and we are now only a few steps away from the first Treg injection in this setting. However, several questions still remain to be addressed including the quantity

of Tregs needed for an efficacious dose, the timing and number of injections and the migration of these cells upon adoptive transfer before the enigma of tolerance induction can be decoded. These factors are discussed in detail in section 6.2.

In addition, in view of the wealth of animal data from our laboratory (Sagoo et al. 2011, Putnam et al. 2013, Xiao et al. 2014) and others in support of the importance of antigen specific Tregs in the setting of solid organ transplantation, chapter 5 outlines the GMP compatible manufacture of antigen specific Tregs with an assessment of both the *in vitro* and *in vivo* function of these cells.

Chapter 5

Clinical Grade Manufacturing of Human Alloantigen-reactive Regulatory T cells for Use in Transplantation

5.1. Introduction and Objectives

Bearing in mind the importance of antigen specific Tregs in the transplant setting and recent reports highlighting the utility of CD40 activated B cells in the induction and expansion of Tregs *in vitro* (Tu et al. 2008, Zheng et al. 2010), this chapter discusses the development of a GMP compatible protocol, for the generation of Tregs with direct allospecificity, using CD40L B cells as APCs with an assessment of their *in vitro* and *in vivo* function. The hypotheses being investigated in this chapter are:

a. Clinical grade Tregs with direct allospecificity can be manufactured using CD40L B cells.

b. Human Tregs with 'direct' allospecificity are more potent as compared to polyclonal Tregs at preventing rejection of human skin in a humanised mouse model.

The objectives of this chapter:

1. To expand B cells, using a GMP compatible CD40L-expressing K562 cell line.
2. To demonstrate the GMP compatible manufacture of alloantigen specific Tregs, using CD40L-stimulated B cells.
3. To test the potency of the antigen specific Tregs as compared to polyclonal Tregs in averting alloimmune mediated skin damage using a humanised mouse model of skin transplantation.

CLINICAL GRADE MANUFACTURING OF HUMAN ALLOANTIGEN-REACTIVE REGULATORY T CELLS FOR USE IN TRANSPLANTATION

SAFINIA *et al.*

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Brief Communication

Clinical Grade Manufacturing of Human Alloantigen-Reactive Regulatory T Cells for Use in Transplantation

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Regulatory T cell (Treg) therapy has the potential to induce transplantation tolerance so that immunosuppression and associated morbidity can be minimized. Alloantigen-reactive Tregs (arTregs) are more effective at preventing graft rejection than polyclonally expanded Tregs (PolyTregs) in murine models. We have developed a manufacturing process to expand human arTregs in short-term cultures using good manufacturing practice-compliant reagents. This process uses CD40L-activated allogeneic B cells to selectively expand arTregs followed by polyclonal restimulation to increase yield. Tregs expanded 100- to 1600-fold were highly alloantigen reactive and expressed the phenotype of stable Tregs. The alloantigen-expanded Tregs had a diverse TCR repertoire. They were more potent than PolyTregs *in vitro* and more effective at controlling allograft injuries *in vivo* in a humanized mouse model.

Keywords: Cellular therapy, clinical application, regulatory T cells, tolerance induction

Abbreviations: arTreg, alloantigen-reactive Tregs; CD40L-sBc, CD40L-stimulated B cells; CFSE, carboxyfluorescein succinimidyl ester; DAPI, 4-6-diamidino-2-phenylindole; DC, dendritic cells; FACS, fluorescence-activated cell sorting; GMP, good manufacturing practice; GvHD, graft-versus-host disease; INFγ, interferon gamma; MLR, mixed lymphocyte reaction; PBMCs, peripheral blood mononuclear cells; PolyTregs, polyclonally expanded Tregs; Tconv, conventional $CD4^+$ T cells; Treg, regulatory T cells; TSDR, Treg-specific demethylated region

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Introduction

Regulatory T cells (Tregs) are essential for self-tolerance (1). In experimental models of transplantation, Tregs are necessary and, under certain experimental conditions, sufficient in establishing transplantation tolerance (2–5). Three Phase I trials evaluating the safety of Treg cell therapy in graft-versus-host disease (GvHD) have been reported and all showed minimal toxicity and suggested possible efficacy (6–8). A Phase I trial of Treg therapy in children with new-onset type 1 diabetes also showed slower disease progression without serious adverse events (9). These findings inspired many to consider applying Treg therapy to solid organ transplantations so that immunosuppression can be minimized or withdrawn.

Alloantigen-reactive Tregs (arTregs) are more effective than polyclonally expanded Tregs (PolyTreg) in inducing tolerance in experimental models of transplantation (10–12). We have estimated that the numbers of Tregs needed for efficacy for humans are in the range of several billion for PolyTregs and 10 times less for arTregs (13). Several approaches have been reported for selective expansion of human arTregs (12,14–16), and none has demonstrated expansion under good manufacturing practice (GMP)-compliant conditions. In this study, we report a robust process for manufacturing clinical-grade human arTregs.

Methods

Cells

Normal donors were consented for whole blood donation. Alternatively, de-identified apheresis products from normal donors were obtained from the UCSF Blood Center. Peripheral blood mononuclear cells (PBMCs) were isolated as described previously (17) and used fresh or after cryopreservation in CryoStor CS10 freezing medium (BioLife Solutions, Bothell, WA). Spleens were from cadaveric organ donors with research consent. All procedures were approved by the authorities at UCSF and King's College London.

Generation of CD40L-expressing K562 cells

Lentiviral vectors encoding human CD40L (NM_000074), CD64 (BC032634), DRA (BC071659) and DRB0401 (18) were produced, and transduction and cloning were performed as previously described (19,20). Stable expression of transduced genes was verified by flow cytometry using antibodies to CD40L (TRAP1), HLA-DR (G46-6) and CD64 (10.1).

Generation of CD40L-stimulated B cells (CD40L-sBc)

B cells were enriched from PBMCs or spleens using the untouched B cell enrichment kit (Invitrogen, Carlsbad, CA), and cultured with irradiated 3T3-CD40L cells (40 Gy) as described (21). The CD40L-sBc were irradiated (30 Gy) and used to stimulate Tregs or cryopreserved in CryoStor CS10 until use. For GMP-compliant expansions, B cells were purified using CD19 positive selection on a CliniMACS (Miltenyi Biotech, Auburn, CA), stimulated with irradiated K562-CD40L cells (100 Gy) in transferrin-containing X-VIVO15 medium (Lonza, Walkersville, MD) supplemented with 10% human AB serum (Valley Biomedical, Winchester, PA), GMP grade IL-4 (Miltenyi) and Cyclosporine A (Teva Pharmaceuticals, North Wales, PA).

Mixed lymphocyte reaction (MLR)

Responder PBMCs labeled with 1.25 μM carboxyfluorescein succinimidyl ester (CFSE; Invitrogen) were stimulated with irradiated allogeneic CD40L-sBc (2 sBc per PBMC) or with irradiated allogeneic PBMCs (5 stimulators per responder). The cultures were harvested after 84–96 h and stained with antibodies to CD3 (clone SK7), CD4 (clone SK3), CD8 (clone SK1), a fixable viability dye, FOXP3 (clone 206D), and HELIOS (clone 22F6). Flow cytometry was performed on Fortessa (BD Biosciences) and analyzed using FACSdiva (BD Biosciences) or FlowJo software (Treestar, Ashland, OR).

Treg expansion

Tregs were isolated using a BD FACSAria II (BD Biosciences) based on the phenotype of $CD4^+CD127^{lo/-}CD25^+$ and PolyTreg expansions were performed as previously described (17). The clinically compliant sorting utilized GMP mAbs generated and provided by Noel Warner (BD Biosciences). For arTreg expansions, the cultures were maintained in OpTmizer Medium (Invitrogen) supplemented with GlutaMAX (Invitrogen), Penicillin/Streptomycin and 2% human AB serum or in X-VIVO15 medium with 10% human AB serum. Fluorescence-activated cell sorting (FACS) purified Tregs were mixed with CD40L-sBc at a 4:1 sBc to Treg ratio. The cultures were maintained with 300 IU/ml human IL-2 until day 9 or 11, when the cells were restimulated with new irradiated sBc or with GMP-grade anti-CD3 and anti-CD28-coated (anti-CD3/CD28) beads at a 4:1 sBc to T cell or 1:1 bead to T cell ratio. Cultures were fed 3 days later and harvested on day 5 after restimulation. Viability of the cells was assessed using trypan blue exclusion.

Flow cytometry

Phenotype of expanded Tregs was assessed using the following three flow cytometric panels: (1) CD8 (clone SK1), CD4 (clone SK3), CD3 (clone SK7) and CD19 (clone SJ25C1); (2) CD4, CD62L (clone SK11), CD27 (clone L128) and FOXP3 (clone 206D; BioLegend, San Diego, CA); and (3) CD4, CD25 (clone 2A3), HELIOS (clone 22F6; BioLegend) and FOXP3. For some experiments, interferon gamma (IFNγ) production by expanded arTregs were assessed as previously described (22). The CD40L-sBc were stained with antibodies to HLA-DR (clone G46-6), CD80 (clone L307.4), CD86 (clone 2331) and CD19 (clone HIB19). The stained cells were analyzed on a FACSCalibur or AccuriC6 (BD Biosciences, San Diego, CA). All antibodies were from BD Biosciences unless otherwise noted.

Treg specificity assay

Expanded Tregs were labeled with 1.25 μM CFSE and stimulated with allogeneic or autologous CD40L-sBc, anti-CD3/CD28 beads, or left unstimulated in media containing 30 IU/mL IL-2. After 72 h, the cells were collected and stained with anti-CD4 and propidium iodide and analyzed on an AccuriC6.

TCRβ repertoire analysis

Genomic DNA was extracted from 0.25×10^6 to 1×10^6 freshly isolated Tregs and *ex vivo* expanded PolyTregs and arTregs. The DNA was submitted to Adaptive Biotechnologies (Seattle, WA) for survey level TCRβ sequencing. Analyses of the sequencing data including determining the clonality index and repertoire similarities were done using algorithms developed by Adaptive Biotechnologies.

In vitro suppression assays

Titrated numbers of expanded Tregs were mixed with 3×10^4 PBMCs from the Treg donor in V-bottom 96-well plates in triplicates. The cells were stimulated with irradiated PBMCs from the sBc or third-party donors for 7 days, and incorporation of ^{3}[H] thymidine during the final 16–20 h of culture was used to measure proliferation. Cultures containing no Tregs were used as controls.

Treg-specific demethylated region (TSDR) methylation assay: Genomic DNA from 0.5×10^6 expanded Tregs was analyzed using licensed reagents from Epiontis GmbH (Berlin, Germany) according to established protocol (23). Percentages of demethylated TSDR were calculated as: [mean copy numbers of unmethylated DNA/(mean copy numbers of unmethylated + mean copy numbers of methylated DNA)] × 100. For female Tregs, the percentages calculated above were multiplied by 2 to correct for X-chromosome inactivation.

Humanized mouse model of skin transplantation

De-identified human skin was obtained from surgery patients with informed consent. The skin was transplanted onto 8- to 12-week-old BALB/c.Rag2$^{-/-}$γc$^{-/-}$ mice and allowed to engraft for 6 weeks before the recipient mice were injected with 10×10^6 HLA-mismatched CD25-depleted PBMCs. Some mice were co-injected with 2×10^6 PolyTregs or arTregs. Histological analysis of the grafts was performed 6 weeks after PBMC injections. For the total duration of these experiments, 100 μg anti-mouse Gr1 (Bio X Cell, West Lebanon, NH) was injected intraperitoneally every 4–5 days to deplete mouse granulocytes. All procedures were conducted in accordance with institutional guidelines. Frozen sections of human skin grafts were fixed with 5% paraformaldehyde and stained with antibodies against human antigens ki67 (cat. # ab15580; Abcam, Cambridge, MA), CD45 (clone HI30; eBioscience), CD3 (cat. # A0452; Dako, Carpenteria, CA), FOXP3 (clone 259D/C7; eBioscience), involucrin (clone SY5) and CD31 (cat. # ab28364; Abcam), followed by incubation with appropriate fluorochrome-conjugated secondary antibodies and mounted with Prolong Gold Anti-fade Reagent with 4-6-diamidino-2-phenylindole (DAPI; Invitrogen). Quantitative assessment of immunofluorescence results was done by counting four to six nonoverlapping fields preformed by an individual blinded to the treatment conditions.

Statistics

Statistical analyses were performed using GraphPad Prism version 5.00 (GraphPad Software, San Diego CA).

Results

CD40L-sBc are potent stimulators of arTregs

Using a one-way MLR, we found CD40L-sBc were markedly more potent than PBMCs at stimulating proliferation of $CD4^+$ T cells, $CD8^+$ T cells and $CD4^+FOXP3^+HELIOS^+$ Tregs (Figure 1A and B). To determine if the proliferation was in response to alloantigens expressed on CD40L-sBc,

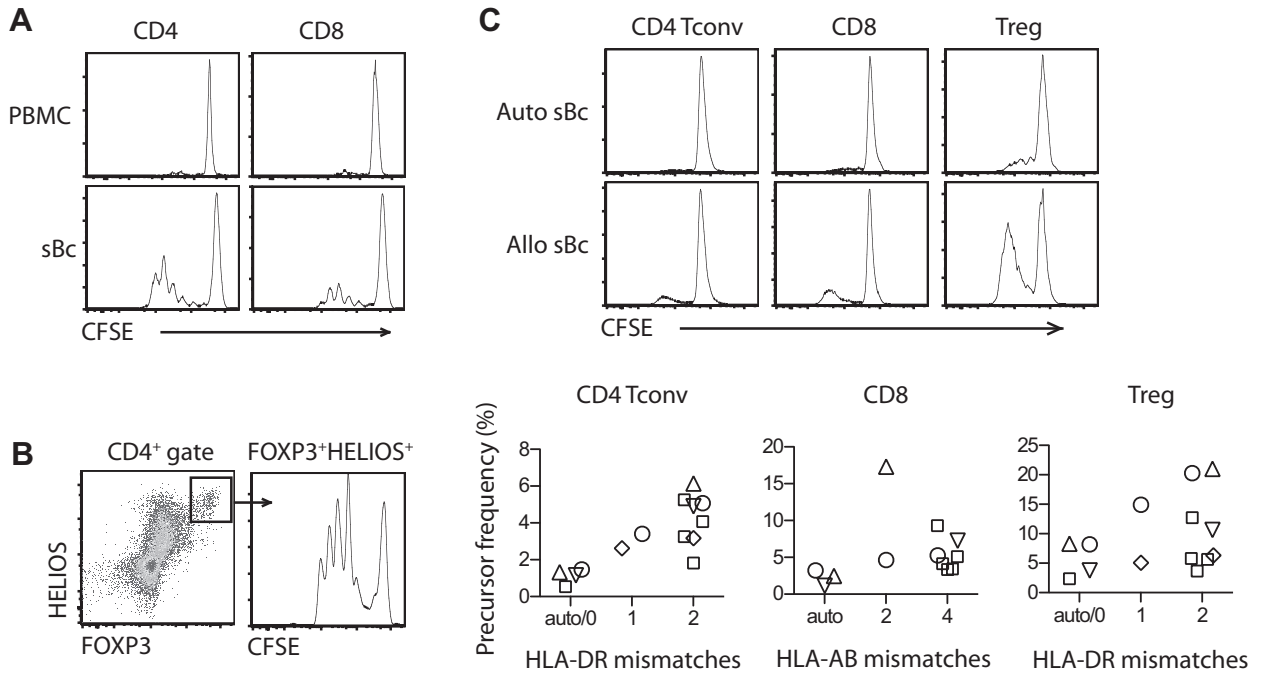

Figure 1: CD40L-sBc potently stimulate T cell proliferation. (A and B) PBMC and CD40L-sBc from the same donor were compared for their ability to stimulate proliferation of alloreactive T cells in a one-way MLR. The responder PBMCs were labeled with CFSE before MLR and the cultures were harvested on day 4 for flow cytometric analysis. Representative CFSE dilution profiles of $CD4^+$ and $CD8^+$ T cells (A) and $CD4^+FOXP3^+HELIOS^+$ Tregs (B) are shown. The data are a representative of at least 10 independent experiments. (C) Autologous CD40L-sBc and allogeneic CD40L-sBc with different degree of HLA mismatches with responder cells were compared in their ability to stimulation proliferation of $CD4^+$ Tconv, $CD8^+$ T cells and Treg cells. Each symbol represents the same responder. Results are a summary of 15 different stimulator and responder combinations. CD40L-sBc, CD40L-stimulated B cells; CFSE, carboxyfluorescein succinimidyl ester; MLR, mixed lymphocyte reaction; PBMC, peripheral blood mononuclear cell; Tconv, conventional $CD4^+$ T cells; Treg, regulatory T cell.

we compared the stimulatory capacity of autologous CD40L-sBc and allogeneic CD40L-sBc with varying degree of HLA mismatches to the responders. We found a trend of higher frequencies of responding $CD4^+$ conventional T cells (Tconv) and Tregs with more HLA-DR mismatches and higher frequencies of responding $CD8^+$ T cells with more HLA-AB mismatches (Figure 1C). These results demonstrated that CD40L-sBc were potent allogeneic stimulators and prompted us to explore the utility of CD40L-sBc in selective expansion of arTregs.

Generation of GMP-compliant CD40L-expressing cells

A GMP-compatible human CD40L-expressing cell line, KT64.CD40L.HLADR0401 (abbreviated as K-CD40L), was generated to enable manufacture of clinical-grade arTregs. We used lentiviral transduction to express CD40L in the myeloleukemia cell line K562, which has been used as cancer vaccines and artificial antigen presenting cells for clinical applications (24–27). The additional CD64 and HLADR0401 genes were intended for other applications and do not interfere with CD40L stimulation of sBc. Two rounds of stimulation with the K-CD40L cells on days 0 and 7 and a constant supply of IL-4 led to 10- to 50-fold expansion of purified B cells (Figure 2A). When compared to freshly isolated B cells, the CD40L-sBc expressed significantly higher levels of HLA-DR, CD80 and CD86 (Figure 2B and C), consistent with their enhanced potency in stimulating T cells.

CD40L-sBc robustly induce arTreg expansion

We have previously reported that polyclonal expansion of FACS purified $CD4^+CD127^{lo/-}CD25^+$ Tregs using two rounds of stimulations (days 0 and 9) with anti-CD3/CD28 beads (17). For expanding arTregs, we compared two rounds of stimulation with CD40L-sBc versus primary CD40L-sBc stimulations followed by anti-CD3/CD28 re-stimulation. Two stimulations with CD40L-sBc led to 50- to 300-fold expansion of Tregs (Figure 3A), similar to that achieved when CD40L-sBc were replaced with beads during restimulation (Figure 3B). Tregs expanded either way were highly reactive to the sBc used for their expansion (Figure 3C). We decided to use bead restimulation for arTreg expansion for the ease of standardization and implementation.

One unit of blood yields an average of 5 million Tregs after FACS purification. With 50- to 300-fold expansions, we would be able to produce between 250 million to 1.5 billion arTregs,

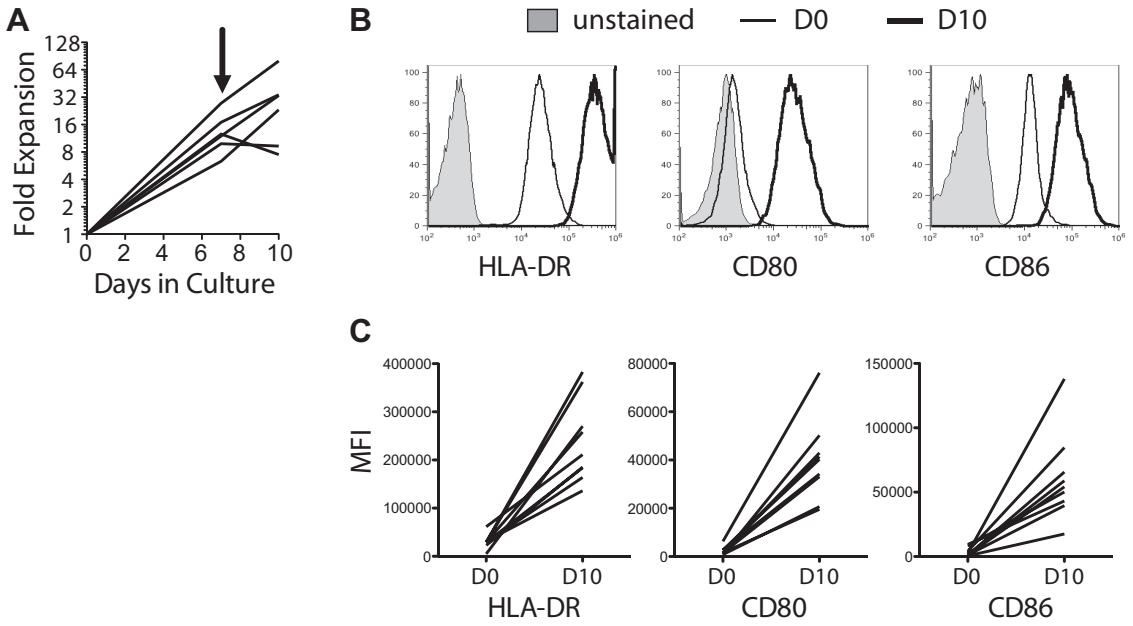

Figure 2: Generation of CD40L-sBc using K-CD40L cells. (A) The expansion of purified B cells in the 10-day culture is shown. The arrow indicates the time of restimulation. (B and C) Expression of HLA-DR, CD80, and CD86 in freshly isolated B cells and day 10 CD40L-sBc was compared using flow cytometry. Sample overlay histograms are shown in (B), and charts summarizing results from independent experiments are shown in (C). The data are summary of six independent experiments. CD40L-sBc, CD40L-stimulated B cells; K-CD40L, CD40L-expressing cell line; KT64.CD40L.HLADR0401; MFI, mean fluorescence intensity.

which may fall short of our estimated efficacy dose (13). We therefore explored conditions to improve arTreg expansion. The CD40L-sBc-stimulated Tregs continued to cluster and blast on day 9 after stimulation (Figure 3D), suggesting that the Tregs were still activated and might undergo activation-induced cell death if restimulated at this time. Delaying restimulation until day 11 when the cells appeared more rested (Figure 3E) consistently improved overall expansion (Figure 3F). The source of the anti-CD3/CD28 beads also affected the rate of Treg expansion (Figure 3G). In contrast, we found that variation in HLA-DR, CD80 and CD86 expression on CD40L-sBc did not correlate with arTreg expansion (Figure 3H), suggesting that the potency of the CD40L-sBc was not strictly correlated with the absolute amount of these molecules as long as a threshold was met. Overall, by optimizing restimulation timing and restimulation regents, arTregs routinely expanded 100- to 1600-fold.

In vitro characterization of arTregs

High-throughput TCR sequencing was used to compare the repertoires of freshly isolated Tregs and various expanded Tregs. arTregs were less diverse than freshly isolated Tregs and PolyTregs (Table 1), consistent with selective expansion using the CD40L-sBc stimulation. arTreg repertoire remained diverse with cumulative frequencies of top 10 clones representing less than 7% of the total repertoire and very low clonality indexes. We found 85% TCR repertoire similarity between the Tregs after primary CD40L-sBc stimulation and after additional anti-CD3/CD28 bead restimulation (Figure 4A). Consistently, arTregs expanded with two rounds of CD40L-sBc or primary CD40L-sBc and secondary bead restimulation had 93% similarity in TCRβ usage (Figure 4B). These results suggest that polyclonal restimulation did not appreciably alter the arTreg repertoire. Last, Tregs isolated from the same individual expanded using two distinct allogeneic CD40L-sBc have very little overlap in their TCR repertoires (Figure 4C), demonstrating alloantigen selective Treg expansion and effective depletion of nonreactive cells using this protocol.

Tregs expanded with this protocol are on average >95% viable and $CD3^+CD4^+$ with minimal contamination with $CD8^+$ T cells and $CD19^+$ B cells (Figure 5A and Table 2). The majority of the $CD4^+$ T cells were $FOXP3^+HELIOS^+$ and co-expressed CD27 and CD62L (Figure 5B and Table 2), distinct from the pattern expressed by similarly expanded Tconv cells (Figure 5B). The majority of the expanded Tregs had demethylated TSDR (Figure 5C, Table 2) and did not produce IFNγ after TCR or mitogenic stimulations (Figure 5D). These results suggested that arTreg were stable and the *ex vivo* expansion did not lead to increase IFNγ expression as we previously reported (22). To determine the reactivity of the expanded Tregs, we restimulated Tregs harvested on day 16 with CD40L-sBc from the same donor. On average 87.5% (range 72.5–95.2%) of the alloantigen expanded Tregs proliferated in response to restimulation by the same sBc, similar to the proliferation induced using anti-CD3/CD28 beads (average 88.8%, range 73.6–96%), demonstrating that the vast majority of the Tregs were

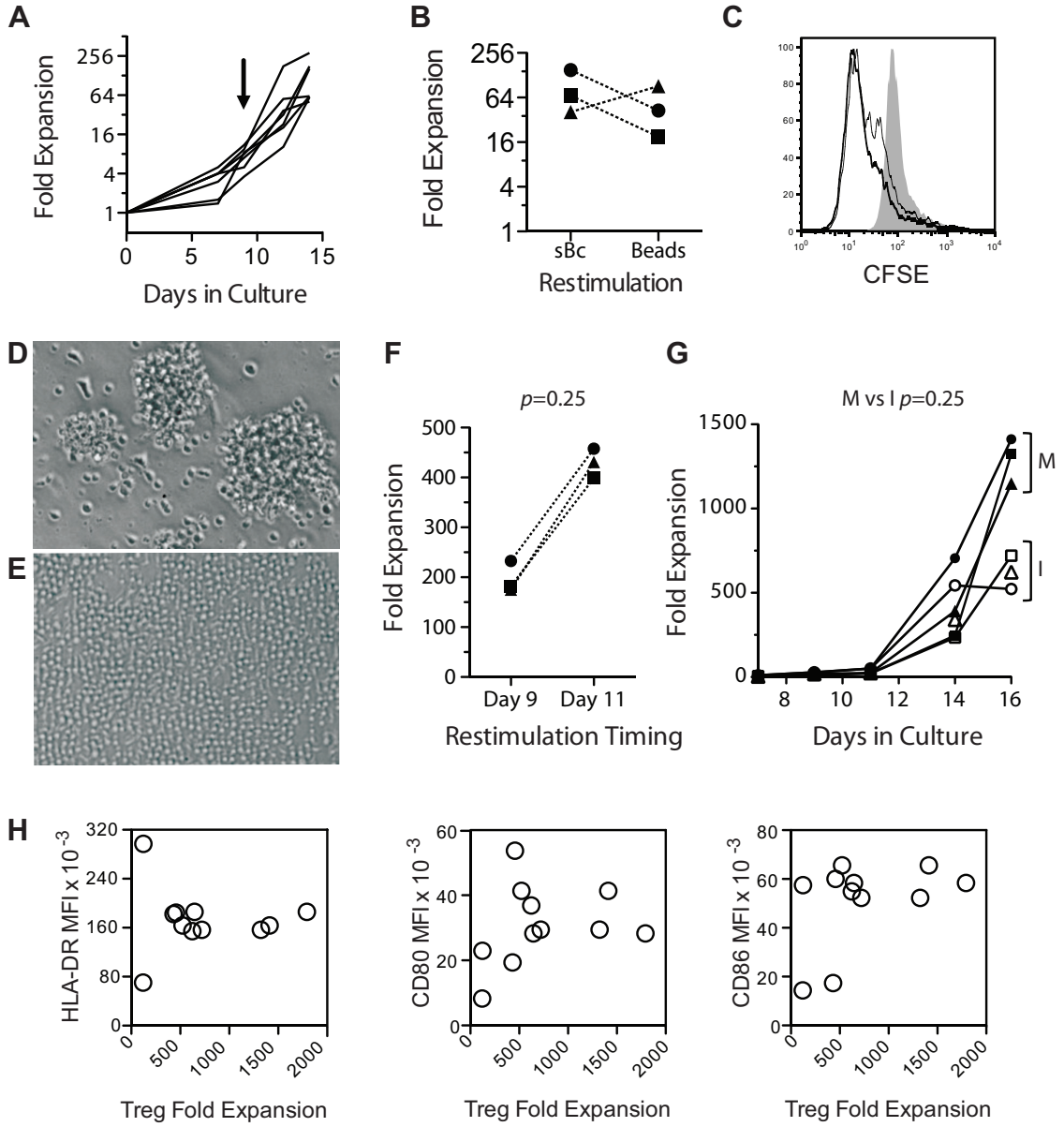

Figure 3: Selective expansion of arTregs using CD40L-sBc. (A) Allogeneic sBc were used to stimulate FACS purified Tregs on days 0 and 9. Fold expansion of Treg in the 14-day culture in six independent experiments is shown. The arrow indicates the time of restimulation. (B) Tregs were stimulated with CD40L-sBc for 9 days and then the cultures were split with half restimulated with CD40L-sBc from the same donor and the other half with anti-CD3 and anti-CD28-coated beads. Fold expansion on day 14 of three independent paired cultures is shown ($p = 0.75$, Wilcoxon matched-pairs signed rank test). (C) Alloreactivity of expanded Tregs was determined by labeling the expanded Tregs with CFSE before restimulation with the same CD40L-sBc used for expansion (thick line), anti-CD3 and anti-CD28-coated beads (thin line) or syngeneic CD40L-sBc (shaded histogram). (D and E) Appearances of Treg cultures on days 9 (D) and 11 (E) after primary stimulation are shown. Data represent results from at least 10 independent cultures. (F) Tregs were stimulated with CD40L-sBc for 9 or 11 days before restimulation with anti-CD3 and anti-CD28-coated beads. The cultures were harvested 5 days after restimulation, and total fold expansions in three paired cultures were compared ($p = 0.25$, Wilcoxon matched-pairs signed rank test). (G) Tregs were stimulated with CD40L-sBc for 11 days before restimulation with anti-CD3 and anti-CD28-coated beads from Invitrogen (open symbols) or Miltenyi Biotec (closed symbols). Cell expansions over time in three paired cultures are shown. Wilcoxon matched-pairs signed rank test was used to compare the difference in total fold expansion on day 16 ($p = 0.25$). (H) XY scatterplots showing a correlation of arTreg expansion and mean fluorescence intensity (MFI) of HLA-DR, CD80 and CD86 expressed on different CD40L-sBc preparations. The data are a summary of 11 independent arTreg cultures. arTregs, arTreg, alloantigen-reactive Tregs; CD40L-sBc, CD40L-stimulated B cells; CFSE, carboxyfluorescein succinimidyl ester; FACS, fluorescence activated cell sorting; Tregs, regulatory T cells.

Table 1: TCR repertoire analysis of Tregs using high-throughput TCR β chain sequencing

1° stim	2° stim	n	% Unique reads	Frequency of top 10 clones (%)	Clonality[1]
None	None	5	10.23 ± 5.39	1.77 ± 0.96	0.037 ± 0.007
Poly	Poly	3	11.36 ± 2.09	1.37 ± 0.71	0.036 ± 0.018
Allo	None	2	5.87 ± 1.45	6.41 ± 2.09	0.122 ± 0.016
Allo	Allo	2	2.34 ± 1.31	5.76 ± 1.82	0.133 ± 0.025
Allo	Poly	4	3.12 ± 1.39	6.86 ± 2.27	0.127 ± 0.027

[1]Clonality is a measurement of repertoire diversity calculated using an ImmunoSeq online analysis tool. The value is between 0 and 1. Clonality of 0 indicates most diverse repertoire, and clonality of 1 indicates monoclonality. Tregs, regulatory T cells.

reactive to the alloantigens expressed by the CD40L-sBc (Figure 5E and F).

Consistent with the phenotype and the enhanced alloantigen recognition, the expanded arTregs were highly suppressive when activated *in vitro* by PBMCs from the same donor as the CD40L-sBc (Figure 5G). arTregs were 5- to 25-fold more potent at suppressing MLR than PolyTregs (Figure 5G), consistent with previous reports of 5- to 32-fold increase in potency by alloreactive Tregs (12,14,28–30). arTreg expanded by restimulation with CD40L-sBc or anti-CD3/CD28 beads had identical suppressive activity (Figure 5G), demonstrating that polyclonal restimulation did not alter their alloreactivity or suppressive activity *in vitro*. In addition, arTregs were 9–27 times more suppressive when stimulated by the relevant PBMC than when stimulated by third-party cells (Figure 5H). Together, our results show that CD40L-sBc expanded Tregs had enriched reactivity and suppressive activity toward the alloantigens expressed by the B cells used for their expansion.

arTregs are superior at protecting skin allografts in vivo

Using our recently described model of alloimmune-mediated injury of human skin allografts (12), we compared the potency of arTregs and PolyTregs. BALB/c.Rag2$^{-/-}$ $\gamma c^{-/-}$ mice were transplanted with human skin from a HLA-DR0401^{+} donor before adoptive transfer of allogeneic PBMC depleted of CD25^{+} cells alone or in combination with different preparations of syngeneic Tregs at a ratio 5:1 effector cells/Treg. PBMC donors were HLA-DR0401^{-} and arTregs from these donors were expanded using HLA-DR0401^{+} CD40L-sBc. Grafts were monitored until rejection or until 6 weeks after PBMC reconstitution when they were collected for histological analysis. Levels of human leukocyte engraftment in spleens were similar in mice that received PBMCs alone or in combination with Tregs (Figure 6A). For the duration of these experiments, all mice maintained stable body weight, suggesting a lack of GvHD (Figure 6B), consistent with our previous report of this model (31).

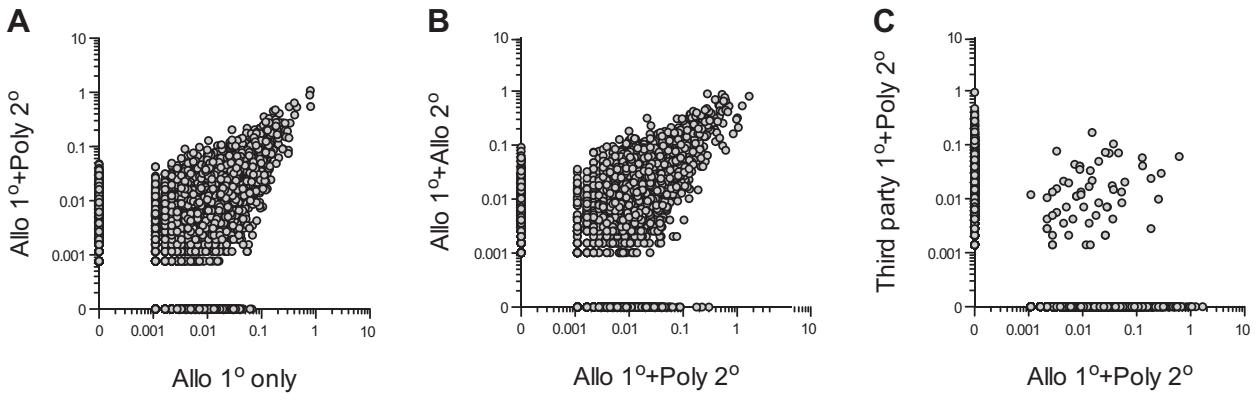

Figure 4: Treg TCR repertoire analyses using high-throughput TCR β chain sequencing. (A) An xy scatterplot was used to compare TCR β chain usage by Tregs after primary CD40L-sBc (Allo) stimulation (x-axis) or after CD40L-sBc (Allo) stimulation and anti-CD3/28 bead (Poly) restimulation (y-axis), showing 85% similarity between the two samples. Each circle represent one unique TCR β chain nucleotide sequence, and data points on the x- and y-axis are present in one sample but absent in the other. The data represent results from two independent experiments. (B) An xy scatterplot was used to compare TCR β chain usage by Tregs after primary CD40L-sBc stimulation and anti-CD3/CD28 bead restimulations (x-axis) and after two rounds of alloantigen stimulations (y-axis) showing 93% similarity between the two samples. The data represent results from two independent experiments. (C) Tregs purified from one donor was split into two equal parts and subjected to primary stimulation with CD40L-sBc from two different allogeneic B cell donors (Allo and Third party) followed by polyclonal restimulation. A comparison of TCR β chain usage by the two arTreg preparations showed 2% overlap. similarity CD40L-sBc, CD40L-stimulated B cells; Treg, regulatory T cell.

Compared to the skin grafts in no PBMC control animals (Figure 6C), grafts in the PBMC alone group showed intense human CD45$^+$ mononuclear cell infiltrates with concomitant increase in keratinocyte proliferation, loss of involucrin and decreased vascularization as indicated by the reduction in clustered CD31$^+$ cells in the dermis (Figure 6D). These changes revealed active inflammation and loss of dermo-epidermal integrity mediated by the allogeneic human leukocytes. All these inflammatory parameters were reduced by co-injection of PolyTregs, correlating with an increase in FOXP3$^+$ cells (Figure 6E). Moreover, skin grafts in the arTreg group were nearly

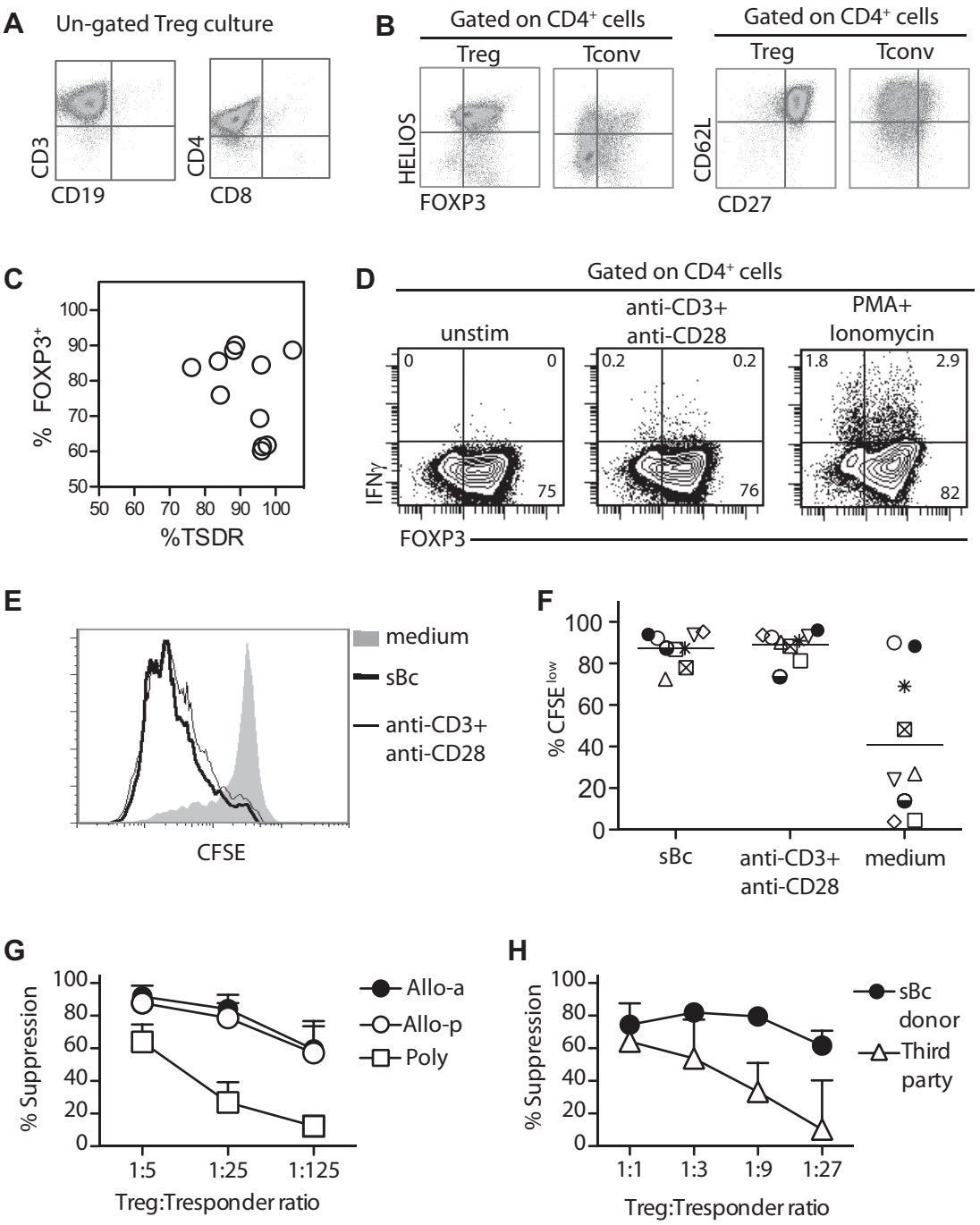

Figure 5:

Table 2: Phenotype of expanded arTregs

	$CD3^+$	$CD4^+$	$FOXP3^+$	TSDR	$HELIOS^+$	$CD62L^+$ $CD27^+$	$CD8^+$	$CD19^+$
Mean	97.1	97.1	83.0	94.0	88.2	85.4	0.5	0.2
SD	2.6	1.9	10.8	15.5	6.6	6.4	0.2	0.2
N	14	14	14	10	14	10	14	14

arTregs, arTreg, alloantigen-reactive Tregs; TSDR, Treg-specific demethylated region.

completely protected from histological features of graft injuries and were indistinguishable from those in control grafts except for the infiltration of $FOXP3^+$ cells (Figure 6F). Quantitative analysis of histological findings demonstrated significant reduction in $Ki67^+$ keratinocytes and an increase in $CD31^+$ cell clusters, correlating with significantly higher $FOXP3^+$ to $CD3^+$ cell ratio in grafts of arTreg-treated mice when compared with the PolyTreg group (Figure 6G). These results suggest improved efficacy of arTregs in protecting allografts *in vivo*.

Discussion

Producing sufficient Tregs during *ex vivo* expansion has been a major challenge in applying Treg therapy to humans (13). By stimulating highly purified Tregs with potently antigenic CD40L-sBc, we were able to achieve 100- to 1600-fold expansion in 16 days. The expanded Tregs had diverse TCR repertoire; retained Treg-specific phenotype were enriched for alloantigen reactivity, and were more potent at suppressing alloimmune responses *in vitro* and *in vivo* when compared to expanded PolyTregs. Critical parameters that contributed to the success of this protocol were the purity of the Tregs at the beginning of the culture, the potency of the CD40L-sBc and the conditions of restimulation.

Naïve B cells failed to induce expansion of Tregs without the addition of anti-CD28 agonist antibodies (30), consistent with the notion that Treg expansion depends on costimulation through CD28 (32). We found that stimulating B cells with CD40L induced nearly 20-fold increase of CD80 and CD86 expression, which may underlie their potency as Treg stimulators. One advantage of using B cells is their relative abundance and ease of expansion when compared to dendritic cells. In the setting of living donor transplant, we estimate that 100 mL of peripheral blood from an organ donor would generate enough CD40L-sBc to expand 5×10^6 Tregs purified from 1 U of blood, which could yield 1 billion arTregs after 200-fold expansion. For deceased donor transplant, donor spleen can be used as a source of CD40L-sBc without prior purification of B cells because their high abundance (data not shown). Our results demonstrate that it is feasible to mass produce highly pure and potent arTregs using GMP-compliant reagents in short-term cultures. Previous reports show Tregs can be expanded from uremic pretransplant patients (33,34). Current efforts are focused on applying this protocol to expand Tregs isolated from pretransplant patient with end-stage organ diseases to enable two planned phase I trials in liver and kidney transplantations. We believe that efficacy of Treg therapy in transplantation depends on the number and quality of Treg products in addition to the timing of Treg infusion and adjunct immunosuppression (5,13).

Figure 5: Phenotype, alloantigen reactivity, and *in vitro* function of Tregs expanded with CD40L-sBc. (A and B) Flow cytometric profiles of ungated (A) and CD4 gated (B) Treg cultures. Data are representative of at least 14 independent experiments. (C) Correlation between percentages of demethylated TSDR and FOXP3 from 11 independent cultures. (D) IFNγ expression by arTregs after 4 h *in vitro* stimulation as indicated. (E) Alloreactivity of Tregs expanded with primary allogeneic sBc stimulation and polyclonal restimulation on day 11 was determined as described in Figure 3B. An example of overlay histogram is shown. (F) A summary of seven independent cultures analyzed as described in (C) is shown. Each symbol represents one independent Treg culture. (G) A summary of *in vitro* suppression by Tregs expanded with two rounds of stimulation with allogeneic CD40L-sBc (closed circles, Allo-a, $n = 3$), allogeneic sBc primary stimulation followed by polyclonal restimulation (open circles, Allo-p, $n = 8$), or two rounds of polyclonal stimulations (open squares, Poly, $n = 5$) is shown. Responders are PBMC from the Treg donor, and stimulators are PBMC from the sBc donor. Data shown are mean ± SEM suppression observed in three to eight independent experiments. Two-way analysis of variance (ANOVA) with Bonferroni multiple comparison test was used to determine the statistical significance of the differences. Suppression at 1:5 ratio by different groups of Tregs is not significantly different. Suppression by PolyTregs is significantly lower when compared to Allo-a Tregs ($p < 0.001$ at 1:25 ratio and $p < 0.01$ at 1:125 ratio), or when compared to Allo-p Tregs ($p < 0.0001$ at 1:25 ratio and $p < 0.001$ at 1:125 ratio). Allo-a and Allo-p Tregs are not significantly different from each other at all ratios. (H) Suppression by CD40L-sBc expanded Tregs stimulated by PBMC from the sBc donors (closed circles) or third-party donors (open triangles) is shown. Data shown are mean ± SEM suppression observed in six independent experiments. Two-way ANOVA with Bonferroni multiple comparison test was used to determine the statistical significance of the differences. Suppression at 1:1 and 1:3 ratios stimulated by sBc and third-party donors is not significantly different. Suppression stimulated by sBc donor at 1:9 and 1:27 ratios is significantly lower when compared to that stimulated by third-party donors ($p < 0.001$ at 1:9 ratio and $p < 0.001$ at 1:27 ratio). arTregs, arTreg, alloantigen-reactive Tregs; CD40L-sBc, CD40L-stimulated B cells; CFSE, carboxyfluorescein succinimidyl ester; INFγ, interferon gamma; PBMC, peripheral blood mononuclear cells; PolyTregs, polyclonally expanded Tregs; Tconv, conventional $CD4^+$ T cells; Tregs, regulatory T cells; TSDR, Treg-specific demethylated region.

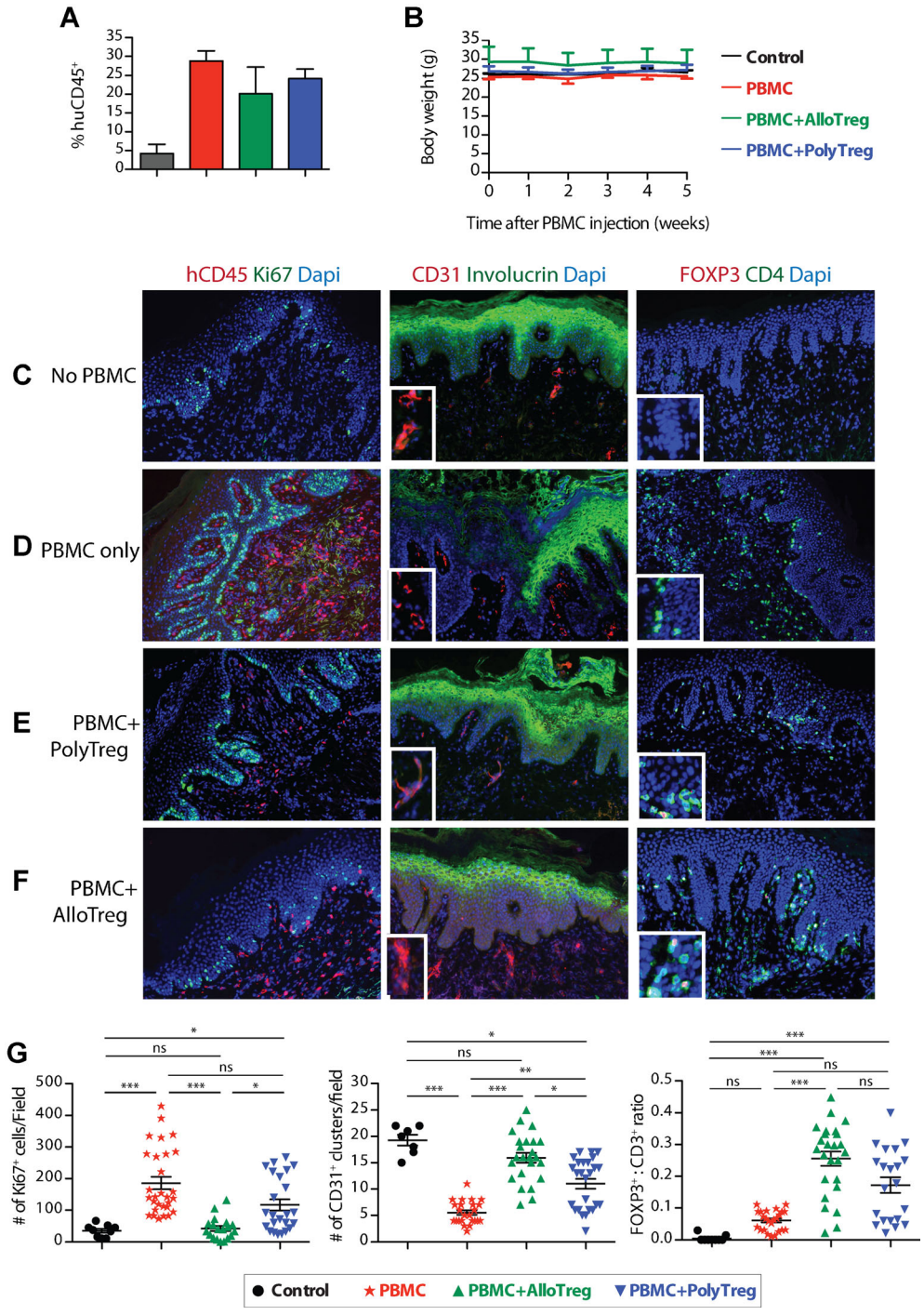

Figure 6: Suppression of skin allograft injury by PolyTregs and arTregs *in vivo* in a humanized mouse model. BALB/c.Rag2$^{-/-}\gamma c^{-/-}$ mice were transplanted with human skin and reconstituted with PBMC allogeneic to the skin donor. (A) PBMC reconstitution was determined at the end of the experiment, demonstrating that co-infusion of Tregs did not significantly alter the extent of PBMC reconstitution. (B) Body weight of the BALB/c.Rag2$^{-/-}\gamma c^{-/-}$ mice in four experimental groups was assessed to determine general health status, demonstrating that PBMC infusion did not induce GvHD. (C–F) Skin graft injury was assessed using three-color immunofluorescence microscopy and representative results are shown. (G) Immunofluorescence micrograph images were analyzed by counting four to six high-powered visual fields per stain for each graft. Quantitative results from four experimental groups were then compared. One-way analysis of variance with Kruskal–Wallis test and Dunn's multiple comparison posttest was used to determine the statistical significance of the differences ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). arTregs, arTreg, alloantigen-reactive Tregs; GvHD, graft-versus-host disease; PBMC, peripheral blood mononuclear cells; PolyTregs, polyclonally expanded Tregs; Tregs, regulatory T cells.

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Authorship Contributions

ALP, NS, AM, ML, MW, GLS, ET, MAM, WL, A Lares, KL, and A Laing performed experiments and analyzed data. RIL, JLR and JAB provided critical advice and review of the studies. QT and GL directed the research and wrote the manuscript together with ALP and NS.

Disclosure

The authors of this manuscript have conflicts of interests to disclose as described by the *American Journal of Transplantation*. JAB and QT are co-inventors on two patents on regulatory T cell therapy. JAB has received reagents and equipment from BD Biosciences in support of developing regulatory T cell therapy.

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Chapter 6

General Discussion

6.1 SUMMARY OF DATA PRESENTED

This thesis has focused on the study of Treg immunotherapy in patients with end stage liver disease, awaiting transplantation. In the UK, ALD is the primary indication for liver transplantation (data from NHS Blood and Transplant Services) and as a result patients with cirrhosis secondary to alcohol (ARC) defined the majority of patients enrolled in this study.

Research in the field of transplantation has focused on effective ways by which to induce tolerance, thereby allowing for the reduction or withdrawal of toxic, non-specific immunosuppressants, currently being used in clinical practice. Whilst there have been many strategies aimed at tolerance induction, cellular therapy with Tregs holds great promise.

The initial aim of this thesis was to characterise Tregs from patients with ARC. However, prior to the *ex vivo* expansion of these cells it was noted that patients with ARC have defects in Treg suppressor function. This novel finding stirred great interest and a programme of work directed as to delineate the mechanism behind this defect, also of importance when considering the clinical translation of these cells.

In this pursuit, cells were subject to an in-depth phenotypic characterization and, in view of studies highlighting the significance of oxidative stress in the development of liver cirrhosis, pathways known to be important in the maintenance of oxidant

homeostasis were also studied. The hypothesis being that dysfunction in the antioxidant pathway correlates with the severity of liver disease in cirrhosis.

In this regard, the role of HO-1 was investigated with data showing that freshly isolated Tregs from patients with ARC had lower expression of HO-1 as compared to age and sex matched HCs.

This thesis also reports the successful GMP compatible isolation and expansion of autologous Tregs from patients with ARC for cell therapy application. In parallel, with the support of the data presented in this thesis, the clinical trial of Treg therapy in liver transplantation, ThRIL, has recently commenced at Guy's Hospital.

Of significance, the rapamycin-based GMP expansion protocol, used in chapter 4, promoted an increase in Treg suppressive function by final harvest, thus guaranteeing that the final product satisfied the set release criteria, essential for the clinical application of these cells in the setting of the ThRIL trial. Interestingly, when correlating HO-1 levels post expansion with Treg suppressive function it was revealed that at final harvest Tregs expressed higher levels of HO-1. Thus, the baseline deficiency of HO-1 expression in Tregs from ARC patients was considered to partially explain their defective suppressor function. This was further supported by data which demonstrated that competitive inhibition of the HO-1 enzyme in HC Tregs decreased their suppressive function. These findings are of relevance not only in defining a mechanism for the Treg defect seen in ARC, but also in aiding the overall understanding of Treg biology.

Moreover, an in-depth phenotypic characterisation of freshly isolated Tregs from patients saw an increased frequency of $CD161^+$ Tregs with a decrease in Tregs, expressing CTLA-4 as compared to Tregs from healthy controls. In addition, post exposure of freshly isolated Tregs from patients to a Th17 skewing environment an increase in $IL17^+FOXP3^+$ cells was evident. However, in chapter 4, *ex vivo* expansion of Tregs in the presence of rapamycin saw a decrease in the frequency of $CD161^+$ Tregs. In line with these findings, culture of Tregs by final harvest in the presence of a pro-inflammatory milieu saw a decrease in $IL\text{-}17^+FOXP3^+$ Tregs with diminished levels of IL-17 production by Tregs.

Lastly, I provide evidence detailing the relative merits of Tregs generated with direct allospecificity, with regards to their potency, as compared to polyclonal Tregs (when injected in equal numbers) in the prevention of alloimmune mediated skin damage in a humanized mouse model of skin transplantation.

In view of Treg immunotherapy in the context of liver transplantation, the next few years will see the results from the ThRIL trial and in parallel, the clinical trial of antigen specific Tregs generated in accordance with the protocol outlined in chapter 5, initiated at UCSF (NCT02188719). Based on my preliminary data detailing that an initial highly pure population of Tregs is required for the generation of antigen specific Tregs, it is important to note that for the ThRIL trial, since GMP compliant sorting technology is not currently available in the UK, the application of antigen specific Tregs is not possible. However, data on the safety and potential efficacy of

polyclonally expanded Tregs (Chapter 4) and antigen specific Tregs (chapter 5) in the setting of liver transplantation will be compared between the two trials.

Of note, in the past, considerable effort has been focused on defining an optimal technique to isolate and expand Tregs from peripheral or cord blood. The data provided in this thesis has shown that expansion of therapeutically relevant numbers of polyclonal or alloantigen specific Tregs is feasible. While the data here is extremely encouraging, there are still many questions yet to be answered. In section 6.2 some of these key issues have been addressed.

6.2 Challenges and future directions of regulatory T cell immunotherapy

The clinical trials of Treg cell therapy initiated in the setting of liver transplantation will report primarily on the safety of the injected cells but will also speculate on their relative therapeutic efficacy, with aims of progressing to larger phase II/III studies in the near future. The success of such trials and the outlook of Treg therapy as an entirety will be defined from effective and informative clinical trial designs with adherence to hard efficacy end points. Thus, key issues will need to be addressed prior to the design of such trials including: adjunct immunosuppressive regimens, the timing and number of injections, the dose of Tregs with the desired specificity and the trafficking properties of the infused cells.

6.2.1. ADJUNCT IMMUNOSUPPRESSION AND TIMING OF REGULATORY T CELL ADMINISTRATION

Despite the initial confidence in adoptive Treg cell therapy as a self-sufficient entity, experimental data has shown that Tregs alone are inadequate in protecting a major MHC-mismatched graft from rejection in a normal, otherwise untreated host.(Wells et al. 1999, Xia et al. 2008). As such adjunct immunosuppressive therapy is needed to create a therapeutic window in which Tregs stand the best chance of inducing tolerance. Moreover, it is now clear that Tregs contribute little, if at all, to direct pathway hyporesponsiveness and, as discussed in chapter 1, it is in fact the indirect pathway of allorecognition used by Tregs for immunoregulation. Thus, drugs that induce a significant reduction in donor-reactive $CD4^+$ and $CD8^+$ T cells, especially those with direct specificity, are likely essential prerequisites for Tregs to induce and maintain graft tolerance (Wells et al. 1999, Xia et al. 2008).

In the clinical setting, most transplant recipients are treated with a combination of immunosuppressive drugs and biological agents to control rejection and/or GvHD responses. Immunosuppressive regimens vary, dependant on the organ being transplanted as well as the individual protocols followed at respective transplant centres. However, bearing in mind the nature of pharmacological immunosuppression it is not surprising that some of these immunosuppressive drugs also antagonise Treg function and survival. At the same time, a select few immunosuppressants have been shown to preserve Treg survival (Demirkiran et al. 2009, Boenisch et al. 2012, Satake et al. 2014), thus, the careful complement of adjunct immunosuppression is of utmost

importance, ensuring the selection of a 'Treg supportive immunosuppressive regimen' to be used alongside adoptive Treg cellular therapy.

In some countries immunosuppressive induction therapy is preferentially conducted with a monoclonal or polyclonal antibody preparation such as alemtuzumab or ATG at the time of transplantation. This treatment markedly depletes most of the leukocyte populations in the peripheral blood. Interestingly, this rather non-specific eradication of leukocytes has the potential to tip the balance in favour of immune regulation by creating a situation whereby regulatory immune cells outnumber the effector cells.

Taking the example of ATG, data indicates that in both adults and children, ATG causes a rapid decrease in naïve $CD4^+$ and $CD8^+$ T cells which usually persist for at least two months (Gurkan et al. 2010). In addition, it has been shown that treatment with ATG is associated with the expansion of $FOXP3^+$ T cells *in vivo* endorsing a shift in the Treg to Teffector ratio. Despite this, memory $CD4^+$ and $CD8^+$ cells are resistant to depletion by ATG and these cell subsets expand over the initial 6 months post transplantation (Gurkan et al. 2010). The fact that memory cells survive deletion may explain why patients do not suffer from opportunistic infections post ATG therapy. However, these cells can contribute to early graft injury/loss and, of significance, these cells are more resistant to the suppressive capabilities of Tregs in comparison to naïve T cells (Yang et al. 2007, Afzali et al. 2011). However, to limit memory T cell expansion (post induction therapy), transplant recipients are maintained on other immunosuppressive drugs, most commonly calcineurin inhibitors (CNI), such as tacrolimus or cyclosporine A. It is well documented that CNIs inhibit the generation and function of Tregs (Segundo et al. 2006, Li et al. 2011). Despite this, animal models, in the context of autoimmunity, have concluded that in order for Tregs to exert their suppressive function, tissue inflammation needs to be controlled

(Korn et al. 2007) and for Tregs to expand *in vivo* and exert their suppressive function, they require a tolerogenic milieu. In support of this, a recent study analysing the dynamics of the alloimmune response *in vivo* demonstrated that in the absence of immunosuppression a rapid invasion of effector cells in the grafts was followed by the delayed arrival of Tregs, which were ineffective at controlling tissue damage (Fan et al. 2010). In contrast, when the recipient mice were treated with anti-CD40L mAb and rapamycin, effector T cell infiltration was delayed and over 30% of the graft infiltrating T cells were Tregs.

Of note, there is good evidence in the literature indicating that rapamycin is superior to tacrolimus for the thymic export and survival of Tregs (Baan et al. 2005, Gao et al. 2007). As such this may suggest that rapamycin is the ideal candidate for short-term therapy post depletion in humans. However, in renal transplantation, rapamycin monotherapy post depletion has been associated with a high risk of acute rejection (Knechtle et al. 2003). Moreover, it is not yet clear whether its concomitant therapy with Tregs would be sufficient to prevent this or whether further immunosuppression will be required in the short term.

The use of combinations of immunosuppressive agents in the clinical setting highlight the challenge associated with designing protocols that include the infusion of Tregs. Thus the competing actions of each immunosuppressive drug may have to be considered together with the key question of the timing of cell injection.

Most of the preclinical Treg therapy studies published to date have administered Tregs prior to or at the time of transplantation, in contrast to studies of autoimmune disease where it has been shown that Treg therapy after disease initiation can

effectively reverse the disease course (Tang and Bluestone 2006). The intensity of the anti-allograft response and the fragility of the transplanted organ may explain the lack of efficacy when Treg infusion is delayed. One caveat to this, however, is in the setting of liver transplantation, where it is speculated that tolerance may be achieved relatively easier because of the inherent resilience and regenerative ability of the liver graft to withstand immune attack. In this regard, histological analysis of the grafts in mouse models that spontaneously tolerated allogeneic liver grafts, showed clear evidence of initial damage, which however, resolved after 2 weeks as the immune response waned (*Bluestone, unpublished data*). As such, in the setting of liver transplantation, there is more flexibility in view of timing of injection.

Another point of consideration, regarding the timing of injection, is the differential use of T cell depleting reagents, such as ATG. In this regard it may also be possible to delay Treg infusion until lymphocyte numbers start to recover two months or more after transplantation. This might tip the balance between Tregs and Teff cells and help promote a tolerant state.

Delaying the administration of Tregs has its own advantages in view of the implementation in the clinical setting, particularly if prior expansion of donor specific Tregs is planned (Sagoo et al. 2011, Putnam et al. 2013).

6.2.2. Location of regulatory T cell function and site of regulatory T cell injection

An additional consideration regarding Treg therapy is the required site of action of Tregs and, consequently, their relative homing properties. In the transplant setting, Treg lymph node homing and their ability to traffic to grafts are both required for their protection against graft rejection (Ochando et al. 2005). Interestingly, in a mouse islet transplant model, it has been shown that therapeutically, Tregs function initially at the graft site (preventing the exit of donor-derived DCs), and then traffic to the draining lymph node and continue to exert their suppressive function there (Zhang et al. 2009). In so doing they prevent the exit and migration of donor derived DCs to the lymph nodes, thereby reducing alloimmune priming. The translation of such a study to the clinic may mean that to ensure that Tregs exert their suppressive function we need to either inject the cells at the graft site or ensure that the cells reach the graft/lymph node either due to their alloantigen specificity or homing receptor expression. Bearing in mind the serious and potentially fatal complications associated with injection of the cells directly at the graft site, i.e. risk of bleeding if cells are injected via the portal vein in the case of liver transplantation, the preferred route of administration is via a peripheral vein.

There is a wealth of reports, together with the data presented in Chapter 5 that donor-specific Tregs are more effective in controlling graft rejection in preclinical models than non-specific Tregs (Trenado et al. 2003, Golshayan et al. 2007, Tsang et al. 2008, Nadig et al. 2010, Sagoo et al. 2011). Moreover, Tregs with direct specificity are known to be more potent in preventing acute rejection early after transplantation,

whilst Tregs with indirect specificity seem to be crucial to prevent chronic rejection (Golshayan et al. 2007, Joffre et al. 2008). As discussed earlier, using antigen specific Tregs in the setting of transplantation has additional advantages in view of offering targeted therapy instead of indiscriminate regulation.

To-date, the generation and expansion of alloantigen specific Tregs has proved to be an arduous task. However, more recently there have been advances in the propagation of Tregs with direct allospecificity (Chen et al. 2009, Feng et al. 2011, Sagoo et al. 2011), with the protocol presented in Chapter 5, providing further data in this regard. However, given the experimental evidence detailing the synergy of direct and indirect Tregs in the setting of transplantation tolerance, considerable efforts have been concentrated on generating and expanding Tregs with indirect allospecificity to further assist in this endeavour (Jiang et al. 2003, Veerapathran et al. 2011). In contrast to the definitive selective expansion of indirect Tregs from the existing repertoire, studies have contended with the possibility of pressuring the expression of TCRs with indirect alloreactivity during expansion of direct alloreactive Tregs so as to generate Tregs with dual specificity, with promising results (Tsang et al. 2008, Brusko et al. 2010).

Contrary to the studies discussed, in GvHD the transfer of Tregs enriched for alloantigen-specificity showed only moderate improved efficacy when compared to a polyclonal Treg cell population (Trenado et al. 2003). In the same setting however, Ukena et al. showed that tolerant patients without GvHD, after haematopoietic stem cell (HSC) transplantation, expressed significantly higher levels of the chemokine

receptors, CCR5 and CXCR3 in Tregs as compared with patients with acute GvHD early after HSC transplantation (Ukena et al. 2011).

Such studies, therefore highlight, that whilst Treg suppressive function is considered a priority, concurrent tissue trafficking of the cells is of equal importance to ensure contact with their target cells.

Therefore, if the Tregs are to be injected via a peripheral vein then it is important that they express the molecules such as CD62L and CCR7 which are crucial for their migration to the lymph nodes and other chemokine receptors e.g. CXCR3 for liver homing (Oo et al. 2010). Moreover, Tregs vary in their expression of trafficking and homing receptors according to their individual histories and state of activation. They have been shown to variously express CCR2, CCR4, CCR7, CCR8, CCR9, CXCR1 and CXCR4 [reviewed in (Ding et al. 2012)]. In addition, it is now known that within the pool of FOXP3 expressing cells, functionally diverse Treg subsets can be identified on the basis of chemokine receptor expression (Duhen et al. 2012). In view of the importance of Treg expression of chemokine receptor and trafficking on their *in vivo* suppression function, efforts have been made to date at understanding the influence of culture conditions on the expression pattern of these receptors on Tregs. In this regard, studies to date have shown that Treg migration can be predicted and engineered by tailored *ex vivo* expansion regimens. For example, rapamycin induces expression of CD62L, CCR4 and CLA on Tregs, favouring skin homing, whilst retinoic acid induces CCR9 and $\alpha 4\beta 7$ that favour migration to the gut (Scotta et al. 2013). This may have important implications for the broader application of Tregs in future trials, such as in the setting of inflammatory bowel disease.

The studies reviewed in this section highlight the importance of antigen specificity and Treg homing receptor expression in the induction of transplant tolerance. With the impending certification of the GMP cell sorter here in the UK, therapeutic approaches in liver transplantation at generating antigen specific Tregs expressing the relevant homing receptor, CXCR3 will soon become a reality. The future will also see studies defining the trafficking patterns of infused Tregs *in vivo*. In this regard, in a recent clinical trial of Treg immunotherapy in Type I diabetes conducted at UCSF, Tregs were labelled with deuterium and their relative homing and survival period was recorded *in vivo* (*Bluestone et al. unpublished data*). In parallel, micro-PET computed tomography fusion has been used clinically to track infused T cells in the body and has further been refined to focus on distinct T cell populations, in particular Tregs (Ribas and Koya 2010). While these technologies are relatively new, the information gleaned from their inclusion in clinical trial protocols of Treg cell therapy will be invaluable, allowing for virtual visualisation of these cells *in vivo*.

The future of cell therapy is also moving in such a way through cellular engineering, introducing concepts of traceable markers, tunable TCRs, chemotactic receptors to synthetic ligands and drug inducible suicidal enzymes (Lim 2010). These designer features would not only allow for the monitoring of infused Tregs, whilst also controlling their activities and trafficking patterns, but also for elimination if and when they become pathogenic (Sato et al. 2007, Guillot-Delost et al. 2008). Nonetheless, further advances in gene therapy would be required for these approaches to move forward, with licensing issues posing their own challenges and hurdles.

6.2.3. Dose of regulatory T cells, number of Injections and monitoring outcomes

Aside from the timing and site of injection what is also of paramount importance is to determine an efficacious dose for Treg cell therapy.

As in the ThRIL trial, the first trials of Treg therapy in solid organ transplantation have started with a dose escalation study to assess the safety and tolerability of Tregs at various doses. It is anticipated that high Treg doses are needed for tolerance induction in view of preclinical studies in mouse models of transplantation where a high ratio of Tregs to Teffectors, in the order of 1:1-1:2, i.e. 33-50% of Tregs, is needed to prevent transplant rejection (Hara et al. 2001, Graca et al. 2002). Moreover, it has been suggested that, combined with ATG induction, a single infusion of 3-5x10^9 Tregs can effectively increase Treg percentage to more than 33% (Tang and Lee 2012). One caveat being the use of or antigen-specific Tregs, where studies have shown that lower numbers are needed to achieve the same functional efficacy as larger numbers of polyclonal Tregs (Tang et al. 2004, Tarbell et al. 2004). Irrespectively, producing such large numbers of Tregs remains technically challenging, especially in view of studies showing a loss of FOXP3 expression after several rounds of stimulation. In this regard, more research is needed to understand Treg commitment and epigenetic regulation of FOXP3 expression so that the mechanisms can be harnessed to stabilise the Tregs.

Another point of consideration is if a single injection of Tregs is sufficient or whether multiple injections are required. This may be determined in larger phase II efficacy studies, where patient outcomes should also be measured and an in-depth patient

monitoring system planned. In this regard, molecular diagnostic tools can be utilised to assess a broad panel of biomarkers, associated with operational tolerance, to serve as surrogate end-points of efficacy (Brouard et al. 2007, Martinez-Llordella et al. 2008, Sagoo et al. 2010).

In this regard, high-throughput, highly-sensitive flow cytometric analysis can also be used to determine if the number of Tregs in the peripheral blood of recipients have increased or relatively quantify the composition of the T cell compartment following the intervention (Hoffmann et al. 2012). Furthermore, the cytokine profile secretion capacity of these cells can be analysed and thus their plasticity evaluated. Investigations using the complementarity-determining region 3 (CDR3) length distribution analysis can be used to explore the diversity of the TCR, in view of studies suggesting that the TCR repertoire might be a good predictor of graft outcome. In this regard, it has been suggested that the majority of kidney transplant patients with chronic rejection have an accumulation of oligo or monoclonal Vβ expansions while operationally tolerant recipients have a TCR repertoire like that of healthy individuals (Miqueu et al. 2010).

As such, a comprehensive immune monitoring plan of patients should be an integral part of a Treg therapy trial in order to gain mechanistic insight on the Treg function in patients. In addition, success in defining optimal ways of measuring tolerance would set the scene for subsequent trials in which accelerated drug minimisation is the principal aim.

6.2.4. STABILITY AND LONGEVITY OF THE INJECTED CELLS *IN VIVO*

For Treg cellular therapy to be a viable therapeutic avenue, two key factors need to be addressed. The first being that, following injection, the Tregs are stable in the graft and draining lymph nodes, despite the inflammation present during transplantation, and secondly whether these cells are either long-lived or able to impart their tolerance to the host immune system.

As the function of Tregs is highly dependent on the constitutively high expression of FOXP3 (Allan et al. 2007) many groups have sought to find ways to stabilise its expression. As discussed in chapter 1, epigenetic modification of the *FOXP3* locus has a major role in controlling *FOXP3* transcription, with demethylation of key regions correlated with suppressive function and lineage stability (Floess et al. 2007). In this regard, *in vitro* treatment with demethylating agents such as azacytidine have shown to promote the stability of FOXP3 expression in Tregs, resulting in the potent ability of these treated cells to protect from GvHD (Sanchez-Abarca et al. 2010). In addition, a recent phase I trial has shown that patients with acute myeloid leukaemia, treated with azacytidine immediately after allogeneic stem cell transplantation had a higher proportion of Tregs as compared time-matched controls (Goodyear et al. 2012).

FOXP3 levels are not only regulated through transcriptional control, but also through post-translational modifications. In the context of transplantation most work has focused on acetylation of lysine residues, which is known to stabilize the FOXP3 protein (Kwon et al. 2012, Liu et al. 2012). It has been shown that inhibiting deacetylation with histone deacetylase (HDAC) inhibitors or genetically removing

Sirtuin-1, a histone and protein deacetylase, leads to an improvement in Treg function and stability, ultimately leading to improved allograft survival (Beier et al. 2011). Thus, future directions of adoptive Treg cell therapy will necessitate further understanding of factors that cause Tregs to lose FOXP3 expression and ways to stabilise its expression.

The question of how long transferred Tregs survive *in vivo* is also of critical importance. It is understood that in order to establish long term dominant tolerance, adoptively transferred Tregs must either survive and expand in the recipient, or be able to induce a tolerogenic phenotype on other T cells, a process known as infectious tolerance (Kendal and Waldmann 2010). It has been shown that some subpopulation of Tregs, such as those producing soluble factors such as TGF-β (Andersson et al. 2008), IL10 and IL35 (Chaturvedi et al. 2011) and the ongoing presence of recipient 'infected' Tregs are required to prevent allograft rejection (Kendal et al. 2011, Gagliani et al. 2013).

In the recent clinical trial of Treg therapy in hematopoietic stem cell transplantation, the transferred cells were no longer detected in the circulation after 2 weeks (Brunstein et al. 2011). Moreover, in the paediatric trial of Treg therapy in Type I diabetes, infusion of $30x10^6$/kg polyclonally expanded Tregs resulted in doubling of the percentage of circulating Tregs and a trend of increase at 2 weeks (Marek-Trzonkowska et al. 2014). In these trials it is not known whether the cells migrated to tissues or died. In this regard, my group have recently used single photon emission computed tomography to image adoptively transferred Tregs in mice and reported that

24 hours after intravenous injection, the cells were primarily localized in the spleen (Sharif-Paghaleh et al. 2011).

Therefore, to maximize the efficacy of Treg therapy efforts will need to focus on finding ways to support the *in vivo* survival, engraftment and function of the infused Tregs. Since Tregs depend on exogenous IL-2 for survival, a suggested approach has been to use low dose IL-2, which lacks the toxicity and immunostimulatory effects of the higher IL-2 doses used to treat cancer patients (Boyman and Sprent 2012). This approach has recently shown to increase the number of Tregs in patients with chronic GvHD (Koreth et al. 2011), supporting the notion that low dose IL-2 may be an ideal adjuvant to adoptive Treg cell therapy, by promoting Treg expansion in an otherwise inflammatory setting.

A final point to consider is how immunosuppression may affect the longevity and function of the transferred cells. As eluded to earlier, there is a general consensus that calcineurin targeted immunosuppression will be detrimental to Tregs (Segundo et al. 2006, Zeiser et al. 2006) as a result there is much interest in tailoring immunosuppression to use drugs such as rapamycin, ATG and/or mycophenolate mofetil.

In this regard, in the ThRIL clinical trial, prior to Treg injection, patients will be maintained on low dose tacrolimus and started on rapamycin. In view of the data presented in chapter 3, it is known that Tregs cultured in the presence of rapamycin have enhanced FOXP3 and suppressive capacity, it is also reassuring that these cells will be injected in a 'Treg supportive' environment, centered around the inclusion of rapamycin. Such a clinical protocol highlights the importance of strategies to tailor immunosuppressive therapy to enhance the Treg longevity *in vivo.* In this regard,

animal models will be required to test strategies such as low dose IL-2 or HDAC inhibitors and if and how these can be combined with Treg therapy to improve its effect.

6.2.5 Anticipated Cost and the Future

At present the cost to manufacture a single 'personalised' injection of Tregs in the GMP facility is over £20,000 in the UK. The data soon emerging on the safety of these cells in the setting of transplantation will provide the basis for progression to a larger phase II/III study. The future progression of the cell therapy programme will also see efforts focused on the optimization of the process development and potential commercialization of the cell based therapies, through collaborations with industry and other organisations. It is anticipated that the future optimization of the manufacturing process for larger scale trials and commercialization would reduce the costs, making this modality of treatment broadly available and applicable in other disease settings.

6.3. Other Novel approaches to tolerance induction

Regulatory T cell therapy, whether it be the adoptive transfer of *ex vivo* expanded cells or induction of Tregs from naïve precursors *in vivo*, has proved its worth as an effective approach to tolerance induction. However these cells are far from the be all and end all, with various other immunoregulatory cellular therapies having also shown considerable promise in inducing tolerance in humans. From a rational

perspective, these can be divided into: strategies aimed at the depletion of donor-reactive lymphocytes, schemes of negative vaccination by injection of non-immunogenic cells, such as modified DCs, and the recent application of mesenchymal stromal cells (MSCs) and regulatory macrophages.

To achieve tolerance in the face of MHC incompatibility it is probable that both T cell deletion and the induction of T cell regulation will be required. Depletion can be either polyclonal, using reagents such as anti-CD3, anti-CD52 (Campath-1), anti-CD25 and anti-CD20 alone, or antigen specific as achieved by haematopoietic chimerism. In the latter approach, the recipient is pre-conditioned with a combination of myeloablative therapy and anti-T cell treatment prior to the infusion of mixed donor and recipient bone marrow, resulting in stable long-term mixed chimerism (Sykes and Sachs 1988). This process tolerises the recipient to alloantigen of the donor and can be followed up by solid organ transplantation. Although, translation of this work in man results in the formation of only a temporary chimerism which appears to cause a wave of deletion of alloreactive T cells in the thymus. This may be accompanied by the thymic selection of a cohort of Tregs that contribute to the subsequent maintenance of tolerance. Needless to say, however, the drawbacks of myeloablative therapy make this an untenable treatment for most patients at present, although a case series published recently demonstrated that a non-myeloablative conditioning regime of cyclophosphamide and anti-CD52 ± anti-CD20 resulted in mixed chimerism that was able to support immunosuppression-free renal allograft transplantation in single-haplotype HLA mismatched donor-recipient pairs. Of the 5 patients treated, 4 retained their grafts whereas one lost the transplant as a result of irreversible humoral rejection in the early post-transplant period (Kawai et al. 2008).

Moreover, several small clinical studies in patients with multiple myeloma and end stage renal failure confirmed the possibility of drug-free renal allograft survival in combination with BMT (Spitzer et al. 2011). These promising results have since provided the impetus for the development of less toxic myeloablative conditioning in solid organ transplantation outside of the setting of haematological malignancy. Several such recent proof-of-concept human studies have demonstrated complete withdrawal of immunosuppressive drugs in a significant proportion of recipients (Scandling et al. 2012, Leventhal et al. 2013, Schneeberger et al. 2013), with long term follow up data currently awaited.

Tolerance induction may also be achieved through the specific targeting and manipulation of DC populations. Although there is no reliable clinically applicable method of depleting donor DCs from grafts, injections of tolerogenic populations of recipient DCs, pulsed with alloantigen, in animal models have yielded encouraging results (Ochando et al. 2006). The concept of tolerogenic DC came from the observation that these cells have an immature phenotypes, holding the capacity to induce antigen-specific tolerance both in central and peripheral lymphoid tissue, (Colonna et al. 2004) However, it was also realised that *in vivo* and in an inflammatory environment there was the risk of these cell adopting a mature phenotype, turning a regulatory response into a potential immunogenic one. In this regard, efforts have focused on developing strategies and using pharmacological agents in order to maintain DCs in an immature state and these generate tolerogenic DCs (Adorini and Penna 2009, Buckland and Lombardi 2009). Additional points of consideration in particular in the transplantation setting is the source of DCs, whether the tolerogenic DC should be of donor or recipient origin (Sun et al. 1996, Beriou et

al. 2005, Peche et al. 2005). Interestingly, my group have shown that donor tolerogenic DCs generated by dexamethasone and vitamin D3 treatment, failed to induce prolongation of skin allograft survival *in vivo* and rather primed the recipient's immune system, despite demonstrating tolerogenic capacity *in vitro (Smyth et al. 2013)*. Such studies merely highlight the challenges currently facing this treatment modality for tolerance induction in the transplant setting.

Of note, however, in the context of the ONE study, the safety and potential efficacy of the administration of recipient tolerogenic DCs in kidney transplant recipients will be addressed (Moreau et al. 2012).

Plasmacytoid DCs (pDC) are a sub-population of DCs, which rapidly produce type I interferon (predominantly IFN-α) in response to viral encounter. Although their antigen presenting capability remains contentious, these cells have several modes of action (Villadangos and Young 2008) including the inhibition of effector T cell responses whilst enhancing the expansion/ induction of Tregs (Nikolic et al. 2009). Furthermore, my group have recently shown the selective depletion of alloreactive T cells, with indirect allospecificity, by targeting the MHC class I monomers to quiescent myeloid DCs *in vivo* (Tanriver et al. 2010). This led to the inhibition of the indirect pathway of allorecognition and the production of IgG alloantibodies, leading to long-term skin graft survival.

An alternative approach, blocking APC co-stimulation, by selective targeting of the CD28-CD80/86 interaction or the CD40-CD154 pathway may also have a role to play in the induction of donor-specific tolerance (Kirk et al. 1999) **(Figure 1.4).**

Furthermore, the selective depletion of B cells through the administration of chimeric anti-CD20 has been considered as yet another mechanism by which to induce tolerance. Of note however, the exact mechanism of action of anti-CD20, apart from B cell depletion, is incompletely understood and diverse activities, including downregulation of the BCR, have been postulated. The exact niche for anti-CD20 has yet to be fully explored, however, most centres reserve its use for induction therapy in highly sensitised individuals to transplant antigens (including ABO blood group incompatible transplants), antibody mediated rejection and for the treatment of rejection resistant to other therapies. The use of anti-CD20 as induction therapy is currently controversial given a report concluding that administration of this agent on the day of transplantation actually increases rates of acute rejection (this study was terminated early as a result) (Clatworthy et al. 2009). Nevertheless, other reports on the use of anti-CD20 in transplantation have been more favourable, with a Swedish study showing a reduction in acute rejection risk when using this agent before transplantation (Tyden et al. 2009). Anti-CD20 is currently under investigation (the RituxiCan study) as a treatment for chronic allograft dysfunction.

It is also important to highlight the growing body of evidence in support of the regulatory activity of a subset of human B cells (Bregs), the $CD19^{+}CD24^{Hi}CD38^{Hi}$ subpopulation, that include immature transitional and shown to reduce $CD4^{+}$ T cell activation at least in part via IL-10 secretion (Blair et al. 2010). Interestingly, in renal transplantation patients, increased frequency of $CD19^{+}CD24^{Hi}CD38^{Hi}$ has been associated with positive outcome (Newell et al. 2010). As such one of the challenges facing B cell depletion therapy is the ability to discriminate regulatory and effector B cells and hence provide B cell targeted therapy. In keeping with this notion, a recent

study using rituximab, a chimeric monoclonal antibody directed towards CD20, reported a higher incidence of acute rejection in the rituximab treated patients as compared to the control group, and those patients who did not receive induction therapy (Clatworthy et al. 2009). Such studies, therefore, demonstrate that total B cell depletion therapy should be avoided and novel approaches considered that manipulate the different B cell subsets.

Over the last few years much interest has focused on the use of mesenchymal stromal cells (MSC) as potential candidates for cellular therapy in solid organ transplantation (Dahlke et al. 2009, Hoogduijn et al. 2010, Roemeling-van Rhijn et al. 2012). MSCs are multipotent stromal cells localised in virtually every tissue and have recently been recognised for their immunomodulatory properties both *in vitro* and *in vivo* (Burr et al. 2013).The precise mechanism by which these cells regulate immune responses is as yet unclear, although the focus of intense research. Whilst traditionally harvested from the bone marrow (Hanley et al. 2013), studies have shown these cells can now be obtained by less invasive approaches, including from adipose tissue (Melief et al. 2013), oral mucosa (Davies et al. 2012), umbilical cord (Karlsson et al. 2012) and dental pulp (*Laing et al. manuscript in preparation)*. The safety of intravenous MSC infusion in autoimmune diseases has already been confirmed in several early phase I clinical trials, with some promise of efficacy (Sun et al. 2009, Duijvestein et al. 2010, Karussis et al. 2010, Ciccocioppo et al. 2011, Bernardo and Fibbe 2012, Connick et al. 2012). However, the observation that these cells can differentiate to sarcoma cells i*n vitro* (Rubio et al. 2013) poses significant safety concerns. As such, these cells are at the heart of intense investigation.

The past few years has also seen efforts focused on developing a cell based medicinal product for use in promoting transplant tolerance in renal transplant patients, using regulatory macrophages (Mregs). The protocol used for the generation of these cells involves the culture of $CD14^+$ peripheral blood monocytes, for 7 days in the presence of M-CSF, 10% human serum and a final 24 hour pulse of IFN-γ (Hutchinson et al. 2011). Mregs derived in this manner have been shown to be homogeneously $CD14^{-/low}$ $HLADR^+$ $CD80^{-/low}$ $CD86^+$ $CD16^-$ $CD64^+$ $TLR2^-$ $TLR4^-$ and $CD163^{-/low}$ (Hutchinson et al. 2011). Moreover, functional analysis of these cells has shown a potent suppressive ability of T cell proliferation *in vitro*, both through IFN-γ induced IDO activity and contact dependent deletion of activated T cells (Hutchinson et al. 2011). Of note, the feasibility of using Mregs to promote allograft acceptance in solid organ transplant recipients has been demonstrated in two clinical trials, the TAIC-I (NCT00223093) and TAIC-II (NCT00223067), in which infusion of donor-derived Mregs, in a range between 7 and 8 x 10^6 cells/kg body weight, were well tolerated and did not raise any safety concerns (Hutchinson et al. 2008, Hutchinson et al. 2008, Hutchinson et al. 2009, Hutchinson et al. 2011). Even still in its infancy, these results are now to be prospectively tested as part of the ONE study.

As outlined in this section, the emerging field of cellular-based medicine offers a broad scope of future immunomodulatory treatment options. Without a doubt, however, preclinical investigations still need to be carried out to answer critical questions addressing bioavailability, *in vivo* distribution, purification, stability,

homogeneity and functional properties of cellular medicinal products must be performed.

6.4. FUTURE WORK

6.4.1. AN IN-DEPTH PHENOTYPIC AND FUNCTIONAL CHARACTERISATION OF REGULATORY T CELLS FROM PATIENTS WITH ALCOHOL RELATED CIRRHOSIS

Whilst the activation of inflammatory responses is a central theme of alcoholic liver injury, excessive generation of ROS has also been reported to play an equally significant role in alcohol induced cellular damage (Zima and Kalousova 2005). In fact, recent studies have suggested a closely-linked relationship between these pathways (Ambade and Mandrekar 2012). Moreover, the contribution of oxidative stress to the chronic inflammatory state, and its steady escalation linked with progression to end stage liver disease, has also been reported with consistent evidence put forward detailing defects in the antioxidant systems early in the course of chronic liver failure (Kirkham 2007, Videla 2009).

In this regard and in view of the expression of HO-1 in many different cell types, induced by ROS, acidosis, proinflammatory cytokines and endotoxins (Pae et al. 2003, Gozzelino et al. 2010), the importance of this enzyme was studied in the context of ARC. The main aim to ascertain whether defects in the expression of this cytoprotective enzyme by patient Tregs can explain the Treg dysfunction reported in patients with ARC.

The future additional series of experiments that are planned are outlined below:

1. To investigate whether defects in the pathways of HO-1 induction can explain the lower expression of this enzyme in patient Tregs **(Figure 6.1).**

   Previous studies have shown that increased gene expression of HO-1 is coordinately controlled by the actions of the transcription factor nuclear factor-erythroid 2-related factor 2 (Nrf2), which binds to the antioxidant response element (ARE) on the promoter regions of the HO-1 gene (Paine et al. 2010). Of note, under basal conditions, the Ketch-like ECH-associated protein-1 (Keap1) forms a complex with Nrf2. The presence of inflammation/oxidative stress stimuli results in a dissociation of Nrf2 from Keap1 and subsequent activation of HO-1 gene expression. As such, Treg cell lysates will be obtained and analysed under basal conditions and in response to lipid peroxidation product 4-hydroxynonenal (4-HNE) known to activate Nrf2 dependent endogenous antioxidant defenses. If shown that 4-HNE induced activation and downstream ARE gene/protein expression is impaired in patient Tregs, this may suggest impaired redox signalling.

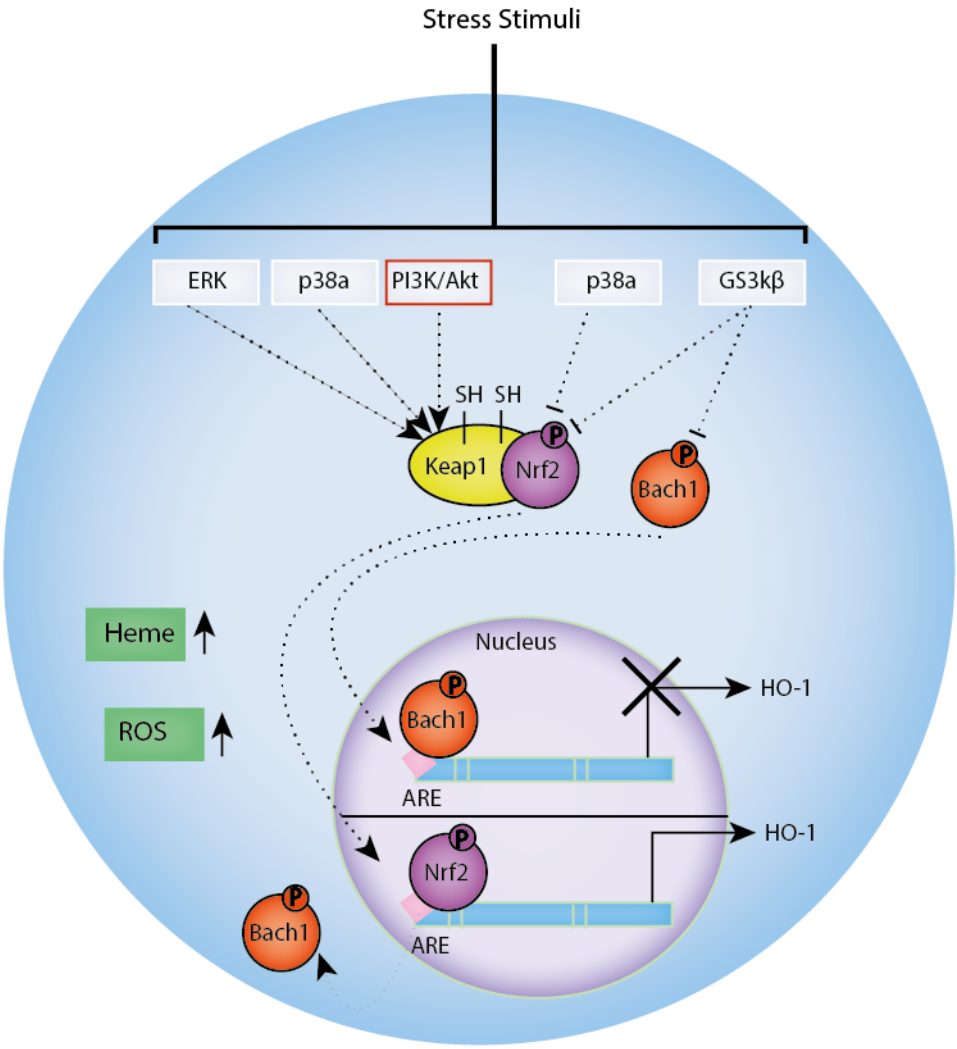

FIGURE 6.1. REGULATION OF HO-1 GENE EXPRESSION.

Diagram depicts the major signalling pathways involved in the regulation of HO-1 gene expression through the transcription factors (TF) Nrf2 and Bach1. These TFs have counter regulatory functions which are controlled by various signalling pathways. At baseline the transcription repressor, Bach1, constitutively binds to the ARE preventing HO-1 gene expression. However, upon activation of certain signalling cascades and high levels of intracellular heme and stress stimuli, the TF Nrf2 dissociates from its complex with Keap1 and translocates to the nucleus, displacing Bach1 at the ARE. As a result HO-1 expression is promoted. While most signalling cascades promote the dissociation of Nrf2, MAPK p38a both activates and inhibits Nrf2 and GSK3β-mediated phosphorylation has been reported to negatively regulate Nrf2 and Bach1. *Abbreviations: ARE-antioxidant response element;, Bach1- BTB and CNC homologue 1; ERK-extracellular-regulated kinase; GSK3β-glycogen synthase kinase-3β; Keap1-Kelch-like ECH-associated protein 1; Nrf2-NF-E2-related factor 2; PI3K- phosphatidylinositol-3 kinase. Adapted from (Paine et al. 2010)*

2. To identify the relationship between the mTOR pathway and the pathway involved in HO-1 induction, in view of the data demonstrating an increased expression of HO-1 in patient Tregs post expansion in the presence of rapamycin.

   The principal mechanism of the immunosuppressant rapamycin, is the direct inhibition of the mechanistic target of rapamycin complex 1 (mTORC1), thereby disrupting the mTOR pathway **(Figure 6.2A).** Inhibition of this pathway precludes the phosphorylation of the downstream protein S6 kinase-1 (S6K1) indirectly activating phosphatidylinositol 3-kinase (PI3K) **(Figure 6.2B).** Following release of its constitutive inhibition, PI3K leads to the activation of protein kinase B (Akt), which in turn promotes the dissociation of Keap1 from the Nrf2 complex. Nrf2 subsequently translocates to the nucleus, binding to the ARE in the promoter region of the HO-1 gene, thus increasing HO-1 expression **(Figure 6.1).**

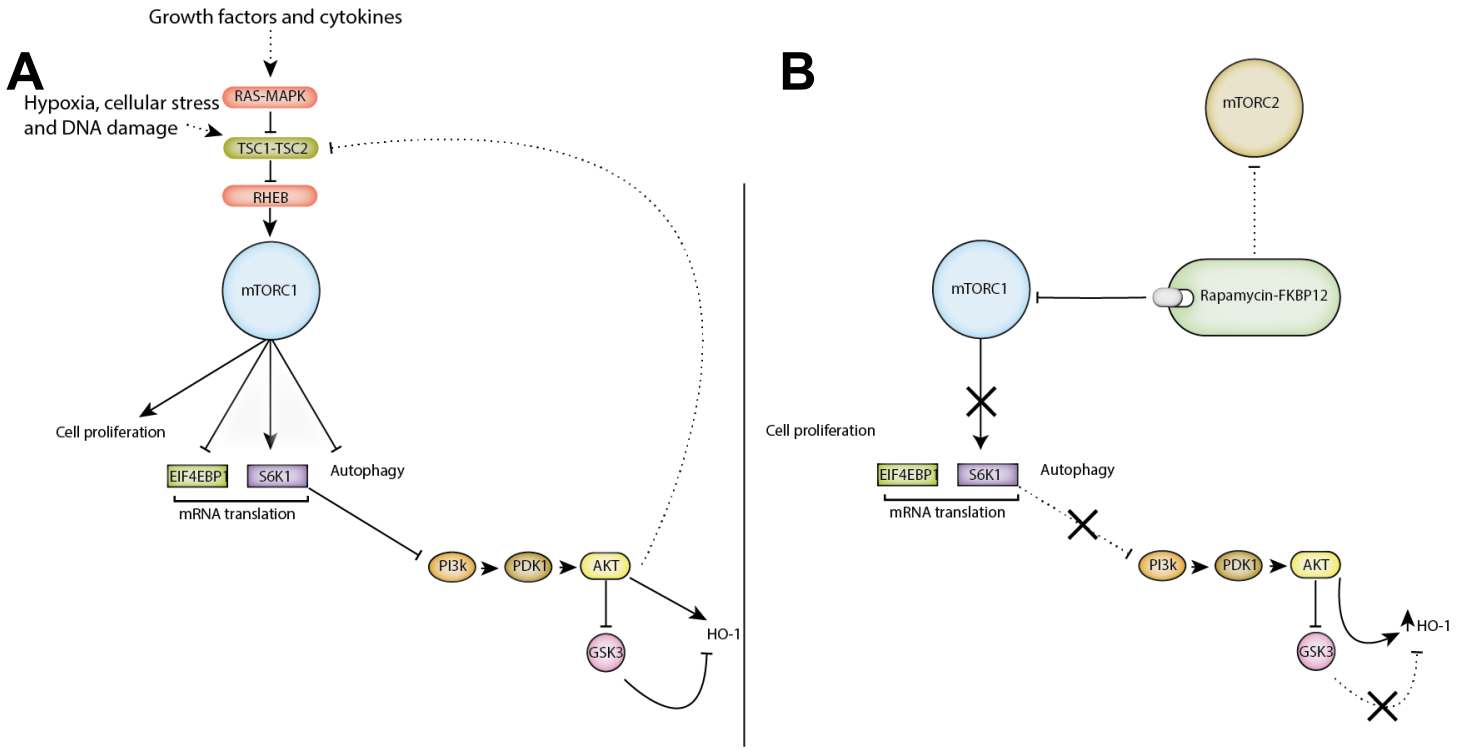

FIGURE 6.2. MAMMALIAN TARGET OF RAPAMYCIN COMPLEX 1 SIGNALLING PATHWAY.

mTOR is a serine/threonine protein kinase that regulates cell growth, proliferation, survival as well as protein synthesis and transcription. It exists in at least two complexes mTORC1, highly sensitive to inhibition by rapamycin, and mTORC2, resistant to rapamycin for reasons that are unknown. **A**.Diverse signals, arising from growth factors, cytokines as well as hypoxia, cellular stress. DNA damage determines mTORC1 activity. These signals mediate their effects through the TSC1-TSC2 complex, which is the main negative regulator of mTORC1. Activation of RAS-MAPK and PI3K-AKT signalling results in inhibitory phosphorylation of TSC2 and removes the repression of RHEB, which is the mTORC1 stimulator. Activated mTORC1 promotes mRNA translation by stimulating S6K1 and inhibiting EIF4EBP1. Activated S6K1 can also feed back to negatively regulate input from PI3K-AKT. In view of the fact the PI3K-AKT signalling cascade is involved in the regulation of HO-1 gene expression, inhibition of this pathway will in turn impair HO-1 gene expression. **B.** mTORC1 is the direct target of the rapamyicn-FK506 complex. This results in inhibition of mTORC1, thus inhibiting S6K1 activation, which in turn leads to the loss of the inhibitory signal on the PI3K-AKT pathway. As such, the activation of the PI3K-AKT signalling cascade results in HO-1 induction either directly or via the inhibition of the GSK3β pathway. It has bee suggested that the PI3K-AKT and GSK3β signalling pathways may have counter-regulatory functions in HO-1 gene regulation. As such, the inhibition of the GSK3β signalling cascade will result in HO-1 induction. *Abbreviations: EIF4EBP1-eukaryotic translation initiation factor-binding protein 1; GSK3-Glycogen synthase kinase 3; HO-1-Heme-oxygenase 1; mTOR-mammalian target of rapamycin; mTORC- mTOR complex); PI3K-phosphoinositide 3-kinase, FKBP12- rapamyicn-FK506-binding protein 1A, 12kDa complex; RAS-MAPK- RAS-mitogen-activated protein kinase; RHEB-RAS homologue enriched in brain; S6K1-Serine/threonine protein kinase ; TSC1-TSC2-Tuberous sclerosis complex 1-2. Adapted from (Thomson et al. 2009).*

3. To delineate the specific mechanism by which Treg suppressive function is linked with the expression of HO-1.

   One potential avenue that may be pursued is the decreased production of IL-10 by Tregs post HO-1 inhibition. A positive feedback circuit between IL-10 and HO-1 has been shown to be functional, amplifying the anti-inflammatory effects of IL-10 (Lee and Chau 2002, Chauveau et al. 2005) **(Figure 1.6)** a process that in part depends on STAT3 activation (Mashreghi et al. 2008). As such it is hypothesized that HO-1 mediated Treg suppression is associated with a release of IL-10 by Tregs. In line with this, De Wilde et al. showed that LPS induced myeloid- derived suppressor cells (MDSCs) inhibited alloimmune mediated skin graft damage via HO-1 and IL-10 production, whereby the inhibition of HO-1 resulted in decreased IL-10 production, abrogating the inhibition of allograft rejection (De Wilde et al. 2009).

   Although, to unearth the definitive mechanism of HO-1 mediated Treg suppression may prove to be an arduous task in view of the individual anti-inflammatory attributes of each of the downstream products of HO-1 (Otterbein et al. 2000) **(Figure 1.6).**

4. To determine whether a defect in HO-1 expression in Tregs can also explain the reduced Treg numbers in patients with ARC.

   The antiapoptotic properties of HO-1 have been widely reported (Brouard et al. 2002, Choi et al. 2004). As such, it can be hypothesized that decreased levels of HO-1 expressed by Tregs from patients with ARC renders these cells

prone to apoptosis, thus reflecting their relative deficiency. Future experiments characterising the susceptibility of patient Tregs to apoptosis will be carried out to confirm/refute this hypothesis and potentially propose an explanation for the low Treg numbers.

It is of importance, however, to also note that in several liver diseases including primary biliary cirrhosis and autoimmune hepatitis, there is a sequestration of Tregs, amongst other infiltrating lymphocytes, in the liver itself ((Sasaki et al. 2007, Sakaki et al. 2008, Speletas et al. 2011)). As such it may be alternatively hypothesized that the decreased number of circulating Tregs reported here is the result of increased migration of these cells into the liver. As the pattern of distribution or function of Tregs in peripheral blood may not necessarily correlate with those found in the liver. As such, further studies will be conducted with an evaluation of Tregs from paired peripheral blood and liver biopsy samples of patients with ARC.

5. In parallel the data presented in chapter 3 also showed that $CD4^{+}CD25^{-}$ Teffector cells from ARC patients expressed comparable levels of HO-1 to age and sex matched HCs, despite autologous Tregs expressing lower levels of HO-1. As such, it can be hypothesized that $CD4^{+}CD25^{-}$ T effectors are protected against apoptosis, as compared to their autologous Treg counterparts. Future experiments will be conducted to test this hypothesis.

   Moreover, although the focus of this thesis has been on Tregs, interestingly, an initial analysis of the T effector cells from patients with ARC showed that these cells were resistant to Treg suppression **(Appendix 2A),** whilst

maintaining similar proliferation potential as $CD4^{+}CD25^{-}$ effectors isolated from HCs **(Appendix 2B).** Future work will also include an in-depth phenotypic analysis of the T effector cells from ARC patients with a study into the mechanism for this resistance. Interestingly, a study by Goodman et al. recently reported that STAT3 phosphorylation mediated the resistance of effector T cells to Treg suppression. In this regard, the next planned experiments will investigate STAT3 signalling in this system (Goodman et al. 2011).

6. Other than the presence of ROS and liver injury mediated by oxidative stress, it is important to highlight that liver cirrhosis has also been associated with a chronic low-grade inflammation (Giron-Gonzalez et al. 2004).

   In this support, there are reports that patients with ARC have elevated levels of pro-inflammatory cytokines in the circulation (Daniluk et al. 2001, Tung et al. 2010). Moreover, the suggestion that in the presence of inflammatory stressors Tregs convert to non-suppressive, pro-inflammatory cytokine-producing cells, whilst retaining FOXP3 expression, (d'Hennezel and Piccirillo 2012, Hamann 2012) has stirred considerable controversy with regard to the inherent stability of these cells. IFN-γ producing Tregs with reduced function have been identified in the peripheral blood of diabetic (McClymont et al. 2011) and multiple sclerosis patients (Dominguez-Villar et al. 2011). This 'Th1-like' Treg phenotype was recapitulated *in vitro* when Tregs from healthy individuals were exposed to IL-12 with the subsequent removal of IL-12 resulting in the reversion back to the classical Treg phenotype (Dominguez-Villar et al. 2011). Such data supports the lineage instability of $FOXP3^{+}$ Tregs,

highlighting their propensity to adopt Th1 and Th17 phenotypes in inflammatory microenvironments.

In this regard, the epigenetic status of the TSDR of the *FOXP3* gene of the freshly isolated Tregs from patients should also be assessed. Key data reported in this thesis supporting this line of investigation, is of an increased propensity of freshly isolated Tregs to produce IL-17 and the increased percentage of $IL17^{+}FOXP3^{+}$ cells, in patients with ARC, after culture in the presence of pro-inflammatory cytokines, as compared to healthy controls.

Moreover, the data presented revealed an increased frequency of $CD161^{+}$ Tregs from ARC patients as compared to healthy controls. Despite recent reports that $CD161^{+}$ Tregs produce IL-17 and yet maintain suppressive function (Afzali et al. 2013, Pesenacker et al. 2013), the role of these cells in ARC has not yet been fully elucidated. The increased frequency of these cells in ARC patients warrants further investigation with regards to suppressive function and cytokine production. In agreement, the data presented further demonstrated that by final harvest, post expansion in the presence of rapamycin, there was a decrease in frequency of $CD161^{+}$ Tregs. Whether this finding also correlates with the increased Treg suppressor function remains to be investigated.

6.4.2. *EX VIVO* EXPANSION OF REGULATORY T CELLS FROM PATIENTS WITH END STAGE LIVER DISEASE; INCREASED SUPPRESSOR FUNCTION AND CLINICAL APPLICATION

Chapter 4 saw the successful isolation and expansion of patient-derived Tregs for clinical application. The data presented provided support to the current evidence (Strauss et al. 2007) that rapamycin protects against plasticity to a Th17 phenotype, whilst maintaining regulatory phenotype and suppressive capacity of the expanded Tregs. It is also encouraging that for the ThRIL trial; patients will be started on a Treg supportive immunosuppressive regimen, one that incorporates rapamycin prior to Treg injection. Despite the rapamycin- based GMP compatible expansion protocol and the clinical protocol, optimised for maintaining a stable Treg phenotype and function *in vitro* and *in vivo*, the stability of the expanded Tregs should be tested by analysing the epigenetic status of the TSDR of the *FOXP3* gene.

6.4.3. CLINICAL GRADE MANUFACTURING OF HUMAN ALLOANTIGEN-REACTIVE REGULATORY T CELLS FOR USE IN TRANSPLANTATION.

Chapter 5 outlines a protocol that can reliably produce large numbers of clinical grade, highly pure, and stable donor-specific Tregs using short-term cultures. In this regard, CD40L stimulated human B cells were shown to be potent APCs in the generation of allogeneic Tregs. This data provided further support to published reports highlighting the utility of CD40 activated B cells in the induction and expansion of Tregs *in vitro* (Tu et al. 2008, Zheng et al. 2010). Traditionally PBMCs (Peters et al.

2008) and DCs (Yamazaki et al. 2003) have been used in the expansion of alloantigen reactive Tregs, with reports of the unparalleled superiority of DCs in this regard (Veerapathran et al. 2011). In addition, an innovative approach to selective expansion of donor reactive human Tregs, using LPS matured human myeloid BDCA-1^{+} (CD1c) DCs has been previously published by my group (Sagoo et al. 2011). However, it yet remains to be determined how B cells and DCs compare in their relative potency as APCs in the generation of Tregs with direct allospecificity. This investigation can be conducted by pursuing the following aims:

1. In-depth characterisation of APCs to allow for the identification of the optimal B cell subset and DC maturation state.

In view of the ever-growing knowledge and understanding of B cell biology, definitive research into their antigen presenting capacities is now all the more plausible with various subsets already identified (Jackson et al. 2008, Perez-Andres et al. 2010, Leandro 2013). As a result, investigation into an optimal B cell subset for the generation of alloantigen reactive Treg may prove to be invaluable.

Moreover the variable maturation states of DCs and their influences on Treg expansion, potency and phenotype will be tested. When considering the clinical translation of this work, it is worth noting that there are already reports of carefully constructed GMP-compliant cytokine cocktails used for the maturation of DCs (Jonuleit et al. 1997, Zobywalski et al. 2007, Navabi et al. 2009).

2. Comparison of the phenotype, function, stability and expansion profile of B cell expanded vs DC expanded Tregs *in vitro*.

Once the optimal APCs have been elucidated additional series of experiments will investigate differences between B cell expanded Tregs vs DC expanded Tregs. Namely, studying the expression of regulatory molecules on Tregs, the chemokine receptor expression and the Treg TCR repertoire. In addition, the antigen specific suppressive function of the B cell expanded Tregs and DC expanded Tregs will be assessed, and the stability of the Tregs studied by analysing the epigenetic status of the TSDR of the *FOXP3* gene.

3. The *in vivo* function of B cell expanded vs. DC expanded Tregs.

It will be pertinent to determine how DC-expanded Tregs compare in preventing transplant rejection *in vivo*, as compared B cell-expanded Tregs. As such, future work assessing Treg homing, survival, function and stability *in vivo* will be assessed, using the well established humanised mouse model of skin transplantation developed in my laboratory. In addition, future work will see investigation into the mechanisms by which one APC is superior over the other with regards to the generation of the optimal antigen specific Tregs.

Other points for consideration in view of the data presented in chapter 5;

1. In chapter 5, it has been shown that B cell expanded Tregs are more potent as compared to polyclonal Tregs at averting alloimmune mediated skin damage. However, in the experiments outlined, similar numbers of both polyclonal and antigen specific Tregs were injected. In view of reports that alloantigen reactive Tregs are 10-100 times more effective at suppressing T effector proliferation to alloantigens than polyclonal Tregs in *in vitro* suppression assays (Peters et al. 2008, Sagoo et al. 2011, Veerapathran et al. 2011), to make a true comparison between their *in vivo* function, 1/100-1/10th of antigen specific Tregs needs to be injected as compared polyclonal Tregs.

2. One of the concerns with the clinical application of polyclonal Tregs in the setting of transplantation is their potential to induce non-specific, 'pan' suppression as compared to the targeted therapy offered by antigen specific Tregs. In future work, using the same humanised mouse model of skin transplantation, mice will be challenged with an infective agent, with levels of immune response mounted compared between polyclonally expanded and antigen specific Treg-treated mice.

6.5. CONCLUSIONS

Clinically, acute allograft rejection can be successfully prevented with the use of immunosuppressive agents. However, such treatment is associated with chronic complications, including, infection, malignancy and renal failure. Long-term allograft rejection, either due to persisting alloantigen recognition via the indirect (and possibly semi-direct) pathway or a failure of tolerogenic mechanisms to fully control alloresponses, remains a fundamental barrier to achieving immunosuppression-free transplantation protocols. Each component of the allorecognition-alloresponse pathway can in theory be manipulated to achieve tolerance.

We are now entering an exciting era in the study of immunological tolerance. Several cellular and molecular strategies of tolerance induction have been developed in non-human transplant models that have shown considerable promise and are just now appearing in clinical trials. As such the recent progress in Treg biology and the successes in the clinical grade manufacture of these cells has seen the start of clinical trials of Treg therapy in solid organ transplantation. Such trials will provide the basis for progression to a larger phase II/III study with a comprehensive patient immune monitoring plan and the use of biomarkers that can predict the successful induction of immune tolerance, allowing for the safe minimisation/withdrawal of immunosuppression. With this all said, it is no secret that the panacea of immunological tolerance in transplantation is now ordained as we take steps ever closer to its fulfilment.

Chapter 7

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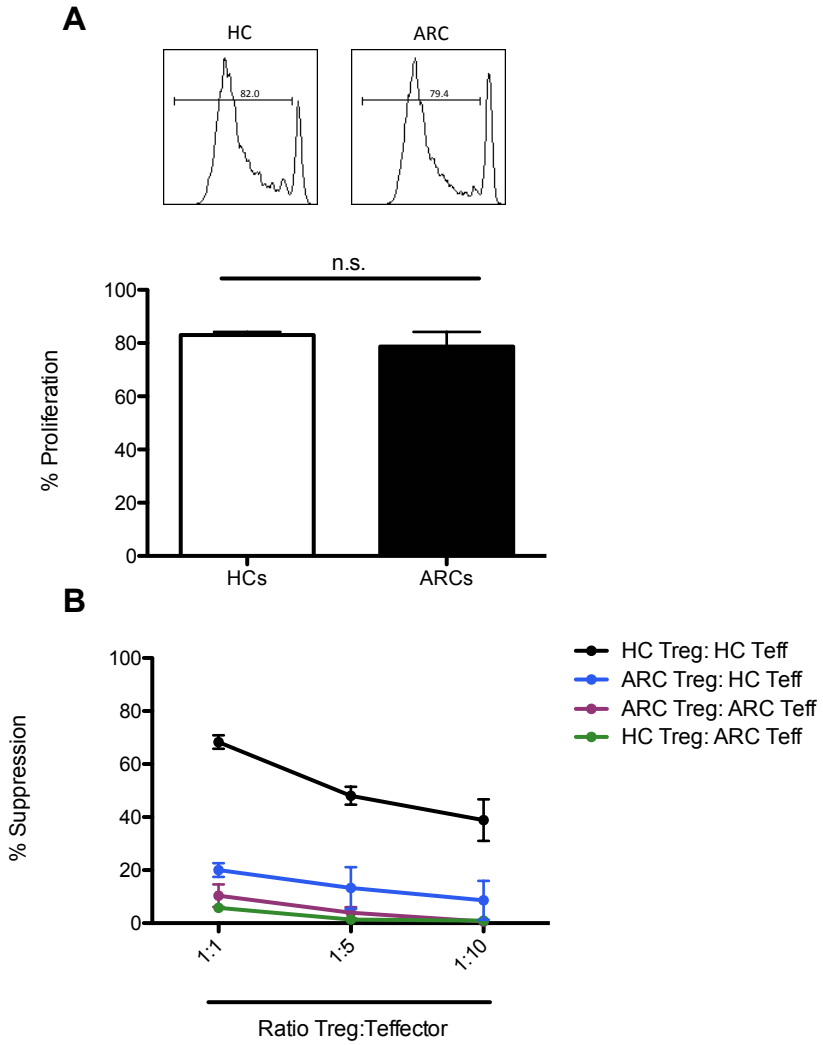
Chapter 8

APPENDICES

Test	Specification
Purity	≥ 60% of entire cell population $CD4^+$ $CD25^+$ FoxP3
Impurities	≤10 % CD8
	≤100 beads per $3x10^6$ cells
	Viability ≥70%
Contaminants	Sterility- no growth after 5 days
	Endotoxin- ≤175 IU/ml
	Mycoplasma - not detected
Potency	≥60% suppression

APPENDIX 1. SET RELEASE CRITERIA FOR CLINICAL TRANSLATION OF FINAL PRODUCT (THRIL TRIAL)

APPENDIX 2.



APPENDIX 2. ASSESSMENT OF T EFFECTOR CELL FUNCTION.

A. Representative histogram and graph comparing the proliferation of $CD4^+CD25^-$ Teffs from 5 ARC patients and 5 HCs. Percentage Teff proliferation was quantified from a CFSE dilution assay. **B.** Graph displaying the relative resistance of ARC $CD4^+CD25^-$ Teffs to Treg suppression. Each line represents the mean percentage suppression following a CFSE dilution suppression assay with $CD4^+CD25^+$ Tregs and $CD4^+CD25^-$ Teffs. The black line displays the mean suppressive function of HC Tregs when co-cultured with autologous HC effectors. The blue line shows the mean suppression of ARC Tregs when co-cultured with allogeneic HC Tregs. The purple line depicts the mean suppression of ARC Tregs when co-cultured with autologous ARC $CD4^+CD25^-$ effectors. The green line shows the mean percentage suppression of HC Tregs when co-cultured with ARC patient $CD4^+CD25^-$ Teffs. n= 5. Treg suppressive function was compared at 3 different Treg:Teff ratios: 1:1, 1:5 and 1:10. n.s.: not significant. Error bars represent SEM.

Appendix 3:

PAPERS PUBLISHED/IN PRESS

- Safinia N, Becker P, Vaikunthanathan T, Xiao F, Lechler R, Lombardi G. Humanized Mice as Pre-Clinical Models in Transplantation. Methods in Molecular Biology (in press).

- Safinia N, Lechler I, Lombardi G. An alliance with Miltenyi Biotec in the fight for tolerance: A venture into no man's land. MACS & more Anniversary Edition 2014

- Putnam AL*, Safinia N*, Medvec A, Laszkowska M, Wray M, Mintz MA, Trotta E, Szot GL, Liu W, Lares A, Lee K, Laing A, Lechler RI, Riley JL, Bluestone JA, Lombardi G, Tang Q. Clinical grade manufacturing and therapeutic advantage of allo-antigen reactive human regulatory T cells in transplantation. Am J Transplant. 2013; 13(11): 3010-20. PMID: 24102808

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- Vossenkämper A*, Blair PA*, Safinia N*, Fraser LD, Das L, Sanders TJ, Stagg AJ, Sanderson JD, Taylor K, Chang F, Choong LM, D'Cruz DP, Macdonald TT, Lombardi G, Spencer J. A role for gut-associated lymphoid tissue in shaping the human B cell repertoire. J Exp Med 2013; 210(9): 1665-74. PMID: 23940259

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- Safinia N, Leech J, Hernandez-Fuentes M, Lechler R, Lombardi G. Promoting Transplantation tolerance; adoptive regulatory T cell therapy. Clin Exp Immunol 2013; 172(2): 158-68. PMID: 23574313. PMID: 23574313

- Safinia N, Afzali B, Atalar K, Lombardi G, Lechler RI. T cell alloimmunity and chronic allograft dysfunction. Kidney Int Suppl. 2010 Dec;(119): S2-12. PMID: 21116312

- Safinia N, Sagoo P, Lechler R, Lombardi G. Adoptive regulatory T cell therapy: challenges in clinical transplantation. Curr Opin Organ Transplant. 2010 Aug; 15(4): 427-34. PMID: 20616725

GRANTS, PRIZES AND AWARDS

- 2015 BRC STEM Early Career Awards.

- 2014 Poster Prize at the British Society of Gastroenterology (BSG). Annual Meeting June 16th-19th, Manchester.

- 2014 The International Transplantation Science Mentee-Mentor Travel Award for the 2014 World Transplant Congress (WTC), July 26-31, 2014, San Francisco CA, USA. Prize for top 3% abstract presented.

- 2013 £2.5 million DPFS/DCS grant awarded by the MRC to conduct 'ThRIL; A 'first-in-human' study, evaluating the safety, tolerability with an investigation into the efficacy of Regulatory T cells (Tregs) in liver transplant recipients' (Co-applicant).

- 2011 £223,490 MRC Clinical Research Training Fellowship grant to fund my PhD, a collaboration between MRC Centre for Transplantation and Institute of Liver Studies (King's College Hospital).

- 2011 $500 Award obtained to attend the FOCIS (Federation of Clinical Immunology Societies) meeting in Washington.
- 2010 €500 Training Award obtained to attend the RISET symposium 'Translating Advances in Transplantation Research'. European Parliament.

- 2010/2011 £10,000 grant obtained from the Biomedical Research Centre (BRC) to conduct research on liver transplantation (part of NIHR/BRC ACF programme).

ORAL AND POSTER PRESENTATION

- Safinia N, Lechler I, Lombardi G. Tregs In Solid Organ Transplantation. Oral presentation at the Frontier's in transplantation meeting. King's College London, Guy's Hospital September 1-2, 2014.

- Safinia N, Vaikunthanathan T, Lechler R, Lombardi G. A GMP Treg expansion protocol restores Treg suppressor function in end-stage liver disease; Implications for adoptive transfer therapy. Oral Presentation at the 2014 World Transplant Congress (WTC), San Francisco, CA, July 26-31, 2014.

- Safinia N, Lombardi G and Lechler I. Approaching Clinical Transplantation Tolerance. Oral Presentation at the 28th European Immunogenetics and Histocompatibility Conference 25th-28th June, 2014, Stockholm.

- Safinia N, Vaikunthanathan T, Lechler I, Lombardi G. The Manufacture of Clinical Grade Regulatory T cells from Patients with End Stage Liver Disease for Cell Therapy Application. Poster Presentation at the British Society of Gastroenterology (BSG) Annual Meeting Manchester 16th-19th June 2014.

- Safinia N, Lechler I, Lombardi G. Regulatory T Cell Therapy; The Journey from Bench to Bedside. Oral presentation at the MRC Head office. Swindon 12th June 2014.

- Safinia N, Blair P, Lechler I, Lombardi G. Alloantigen reactive human regulatory T cells, expanded using the 'optimal' antigen presenting cell: implications for clinical application. Poster presentation at the British Society of Immunology (BSI), December 2013.

- Safinia N, Vaikunthanathan T, Fraser H, Lechler I, Lombardi G. Isolation and expansion of regulatory T cells from liver transplant recipients at GMP standards; Implications for cell therapy application. Oral Presentation at the British Transplant Society (BTS); Bournemouth 2013.

- Safinia N, Blair B, Lechler I, Lombardi G. Alloantigen-Specific Human Regulatory T cells Stimulated with CD40L-Activated Allogeneic B cells Prevent Allograft Damage in a Humanized Mouse Model. Oral Presentation at the British Transplant Society (BTS); Bournemouth 2013.

- Safinia N, Lombardi G, Lechler I. Tolerance in the clinic, where do we stand? Oral presentation at the European Society of Organ Transplantation (ESOT) Basic Science Meeting Paris 2013.

- Safinia N, Lechler I, Lombardi G. Allospecific Regulatory T cells and Prevention of Hepatocyte Rejection. Poster Presentation FOCIS (Federation of Clinical Immunology Societies), Washington 2011.